BLOOD ORGANOCHLORINES, IMMUNE FUNCTION AND HEALTH OF FREE-RANGING NORTHERN FUR SEAL PUPS (*CALLORHINUS URSINUS*)

A

THESIS

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Kimberlee Beth Beckmen, B.S., M.S., D.V.M.

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Abstract

This study examined organochlorine (OC) contaminant levels in blood and milk along with immune function and health of northern fur seals (*Callorhinus ursinus*) from St. George Island, Alaska. This portion of the Pribilof Islands breeding stock has undergone a long-term decline between 4 and 6 % per year for unknown reasons. To examine the possible role of neonatal OC exposure on health, two cohorts of pups (69 total) and 33 matched periparturient dams were captured for blood and milk sample collection. From the second cohort of 49 neonates, 43 were re-sampled 29 to 51 days later. OCs were extracted from whole blood and milk to identify 15 polychlorinated biphenyl (PCB) congeners and 4 metabolites of dichloro-diphenyl-trichloroethane by high performance liquid chromatography. Peripheral blood lymphocytes were isolated and cryopreserved for *in vitro* lymphoproliferative immunoassays. These cellular function assays, along with complete blood cell counts, growth rates and survival through the early developmental period, were used as indicators of health status. Humoral immune function was assessed by *in vivo* antibody responses to tetanus vaccination. Mean blood levels of PCBs were higher in neonate samples than in pups one to two months old. Seven of the eight congeners detected in blood were higher (lipid weight) in neonate blood than in dam blood or milk. First-born neonates were exposed to higher levels of OCs from ingested milk and had higher blood levels of OCs than neonates of older, multiparous dams. Higher OC exposure in neonates was correlated to higher blood OC levels and poorer lymphoproliferative responses as well as lowered serum retinol and thyroxine. Higher proportions of pups born to old dams developed tetanus antibodies

compared to the pups of young dams. Higher OC exposure and poor immune responses in first-born pups may indicate a higher risk of secondary morbidity and mortality than for pups born to multiparous dams but an affect on growth rate or survival to midway through the nursing period was not detected. Evidence of substantial OC contaminant exposure at a critical period of development for the immune system must be considered as a potential contributing factor to reduced post-weaning survival.

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List of Abbreviations

ADL	above detection limit
AGL	average growth in length
AGM	average growth in mass
Ah	*aryl* hydrocarbon
BDL	below detection limit
BMF	biomagnification factor
CB	chlorinated biphenyl
CI	condition index
CPT	cell preparation tube, formerly Leukoprep™
ConA	concanavalin A
DDD	dichloro-diphenyl-diethane
DDT	dichloro-diphenyl-trichloroethane
DDE	dichloro-diphenyl-ethane
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
ECD	electron capture detection
EDTA	ethylenediaminetetracetic acid
ELISA	enzyme linked immunosorbant assay
FBS	fetal bovine serum
FT3	Free triiodothyronine
FT4	Free thyroxine
HCB	hexachorobenzene
Hb	haptoglobin
Hg	hemoglobin
HPLC	high performance liquid chromatography
Ig	immunoglobulin
IUPAC	International Union of Pure and Applied Chemistry
l.w.	lipid weight

MDL	mean detection limit
NK cell	natural killer cell
NMML	National Marine Mammal Laboratory
NWFSC	Northwest Fisheries Science Center
OC	organochlorine
PBMC	peripheral blood mononuclear cells
PBML	peripheral blood mononuclear leukocytes
PBS	phosphate buffered saline
PCDD	polychlorinated dibenzo-*p*-dioxins
PCDF	polychlorinated dibenzo-furans
PHAH	polyhalogenated hydrocarbon
PCB	polychlorinated biphenyls
PHA	phytohemagglutinin
PWM	pokeweed mitogen
RBP	retinol binding protein binding complex
Rrel	relative ratio
SI	stimulation index
TCDD	tetrachlorodibenzo-*p*-dioxin
TEF	toxic equivalency factor
TEQ	toxic equivalency quotient
TSH	thyroid stimulating hormone
TTR	transthyretin
TT3	Total triiodothyronine
TT4	Total thyroxine
w.w.	wet weight

Preface

This study was conceived to emphasize the importance of investigating the potential effects of organochlorine contaminant exposure on a free-ranging wildlife population, beyond the measurement of tissue residues. The clamor for 'ecosystem health assessments', particularly involving declining stocks of marine mammals in the Bering Sea, and the recent advances in the fields of toxicology and immunology, made this study possible. Northern fur seals lend themselves well for field study because of their site fidelity and predictable nursing/foraging behavior while around the Pribilof Islands. The ability to obtain repeat blood samples and the development of field techniques to isolate and cryopreserve lymphocytes was paramount to the immune function and health assessment portions of the study. Advances in reagent and assay development at the Laboratory for Marine Mammal Immunology at the University of California Davis were key in evaluating the immune function after the field season. Of equal importance, the ability to extract trace levels of PCB congeners from whole blood for detection by HPLC, was developed by the Northwest Fisheries Science Center during the course of this study. Blood OC detection is a tremendous advance over the traditional blubber OC residue surveys from a humane and practical standpoint as well as for more realistic assessment of actual OC exposure.

The thesis is comprised of four chapters that will be submitted separately to peer reviewed journals for publication. Chapter 2, "Factors Affecting Organochlorine Contaminant Concentrations in Milk and Blood of Northern Fur Seal (*Callorhinus ursinus*) Dams and Pups from St. George Island, Alaska" is currently in press in *The*

Science of the Total Environment. The manuscript is co-authored by G.M. Ylitalo, R.G. Towell, M.M. Krahn, T.M. O'Hara and J.E. Blake. Chapter 3, "Age-dependent Effects of Organochlorine Contaminant Exposure on Hematological and Immune Functional Assays in Free-ranging Northern Fur Seal Pups", will be revised and submitted to *Marine Mammal Science* as a co-authored manuscript with T.M. O'Hara, J.E. Blake, J.L. Stott and G.M. Ylitalo. Chapter 4, "Association of Organochlorines in Milk and Pup Blood with Serum Retinol and Thyroxine Levels in Northern Fur Seal Pups", will be revised and submitted to *Marine Pollution Bulletin* as a manuscript co-authored by J.E. Blake and G.M. Ylitalo. The last chapter, "Effects of Parity and Dam Mass on Pup Mass, Length, Growth, and Condition in a Cohort of Northern Fur Seal Pups", will be revised and submitted to the *Canadian Journal of Zoology* as a co-authored manuscript with A.W. Trites.

There have been a great number of people involved throughout this project, from initial study design through to final manuscript preparations, and I am grateful to all. I wish to begin by expressing my thanks to Bud Antonelis, Tom Loughlin, and Terry Spraker, for introducing me to the fur seal rookeries of the Pribilof Islands. Their motivation to investigate potential reasons for St. George seal population decline was the seed of all that was to come. Because of the same concerns, Larry Merculief pushed to obtain the funding for a preliminary study, which demonstrated the need and feasibility of this project. For this boost, I am grateful. Logistical support from the following is gratefully acknowledged: Village of St. George; St. George Traditional Council, especially Gilbert Karshevarof; City of St. Paul; National Marine Mammal Laboratory,

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I owe a great debt of gratitude to Mark Bushong, Mike Goebel, Rolf Ream, Jeremy Sterling, Rod Towell, and Sally Troy, whom were all responsible for the hard work of capturing fur seals to order. Rod Towell's determination and patience were so priceless to my success that I can't begin to express my gratitude to him.

I am also indebted to my advisor and mentor, John Blake, who fostered me via the Wildlife Veterinary Residency program. I am especially appreciative to him for guiding me through the bureaucracy of academia and teaching me how to succeed. Thanks to his encouragement, as well as his firm yet gentle ways propelling me forward, I was able to accomplish my goals. Thanks and recognition all around to the members of my committee for their time and efforts on my behalf including Bob Elsner, Erich Follmann, Mike Castellini, and Terry Spraker. A special recognition of appreciation must be given to Todd O'Hara, who was not just a teacher and mentor, but a friend. I could not have achieved the completion of this project and gotten my feet headed in the right direction without his steady guidance, timely critical advise and sense of humor.

Months after the field work was done, the often tedious, but most essential laboratory analyses were carried out with great care by Gina Ylitalo, Jon Buzitis, Larry Hufnagle and Myra Blanchard. All the field work would have meant nothing without the

data, thank you! Additional thanks go to all those who provided welcome advice and assistance along the way including Jeff Stott, Peggy Krahn, John Stein, Dana Thomas, Don King, Andreé Porchet, Ted DeLaca, Teri Rowles, Andrew Trites and anyone I've inadvertently left out.

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I dedicate this thesis to my parents, Carl and Joyce Beckmen, my family and friends for their tremendous support of me on this long and winding road.

And to Mark Justin Bushong, I know your presence still by the faint sound of hummingbird wings.

Chapter 1

Introduction

The northern fur seal, *Callorhinus ursinus*, (Linnaeus 1758) is a sexually dimorphic, polygynous pinniped, the sole member of its genus and classified in the Family Otariidae. This mainly pelagic seal inhabits the north Pacific Ocean from about 35 to 60 degrees north latitude. Breeding rookeries and seasonal haulout areas for more than 72% percent of the world's population are located in the Bering Sea on the two largest Pribilof Islands, St. Paul and St. George, Alaska (Loughlin et al., 1994).

During mid-March pregnant fur seals begin a migration back to the Pribilof Islands from foraging areas in the north Pacific and arrive on the rookeries approximately three to four weeks after the arrival of territorial males in May (Bigg, 1990). Parturition occurs within 1-2 days after arrival (during the period from late June to early August) with the peak of births occurring in early July. A brief estrus occurs on about the 6th postpartum day but embryonic diapause (delayed implantation) lasts until the end of lactation, 120-142 days postpartum. The lactating seal typically alternates 5-11 day foraging trips with 1-3 day nursing bouts until pups spontaneously wean and migrate in October or November (Macy, 1982; Gentry, 1998). During the winter, pregnant fur seals and juveniles of both sexes feed in wide areas of the North Pacific including the continental shelves off North America, Russia and Japan while adult males migrate only as far south as the Gulf of Alaska (Bigg, 1990; Ragen et al., 1995). Weaned pups remain at sea for 22 months before returning to their natal rookery.

The Pribilof stock has undergone constant human exploitation for pelts since its discovery in 1786 until the end of the commercial harvest in 1972 on St. George and 1985 on St. Paul. A small native subsistence harvest continues on the islands, which consists of approximately 2,000 subadult (2-4 years of age) males per year. The current Pribilof stock abundance is less than half of historical levels and is listed as depleted under the Marine Mammal Protection Act (Loughlin et al., 1994; York et al., 1997). Pup production on St. Paul Island has stabilized following a decline of about 7% per year from 1975-1983 (York, 1990). Until 1996, the St. George subpopulation underwent an unexplained decline of 4-6% per year for more than a decade despite protection from exploitation (York et al., 1997). Long-term population trends suggest that the decline was due, at least in part, to increased post-weaning mortality at sea (Trites and Larkin, 1989; Trites, 1992). The cause(s) of the increased mortality is unknown.

Organochlorine (OC) contaminant exposure, including polychlorinated biphenyls (PCBs) and the dichlorophenyl-trichloroethane (DDT) group**,** has been documented in the Pribilof population of northern fur seals through tissue residue analyses, but the biological significance or potential health effects of OC exposure remains to be investigated (Anas and Wilson, 1970a; Anas and Wilson, 1970b; Kurtz and Kim, 1976; Calambokidis and Peard, 1985; Calambokidis, 1987; Bacon et al., 1992; Varansi et al., 1992; Schantz et al., 1993; Mössner et al., 1994; Becker et al., 1997; Krahn et al., 1997; Mössner and Ballschmiter, 1997; Becker et al., 1997). Tables 1.1 and 1.2 summarize blubber OC concentrations from northern fur seals obtained from the literature, but comparisons across studies must be considered cautiously. Analytical techniques vary

between laboratories and monumental advances have been made in techniques over the past 20 years. The older method of reporting PCBs as Aroclors is quite outdated and may report a concentration that is 2 to 4 times the sum PCBs (Turle et al., 1991; Hutchinson and Simmonds, 1994; Newman et al., 1998). Sum PCBs also are reported with varying numbers of congeners detected, usually the congeners in the highest concentrations. Using current methods, in comparison to other Alaskan seals, northern fur seal subadult males have blubber levels of some OC contaminants that are higher than other seal species such as the bearded seal, *Erignathus barbatus*, the ringed seal, *Phoca hispida* and the harbor seal, *Phoca vitulina* (Becker et al., 1997).

Organochlorine compounds are ubiquitous environmental contaminants that are anthropogenic, persistent and have become globally distributed via atmospheric circulation leading to deposition even in remote arctic and subarctic ecosystems far from the sites of original release (Risebrough et al., 1968; Born et al., 1981; Tanabe et al., 1983; Tanabe et al., 1984; Muir et al., 1988; Tanabe and Tatsukawa, 1991; Barrie et al., 1992; Muir et al., 1992; Norstrom and Muir, 1994; Addison and Smith, 1998; Muir et al., 1988; Barrie et al., 1992). The open ocean has become a global sink for PCBs. As of 10 years ago, only 31% of the known world production of PCBs had been released into the environment and environmental levels, especially in remote areas, are unlikely to decline in the near future (Tanabe, 1988). OCs are lipophilic and tend to accumulate in top predators. This has been documented in the tissues of a wide variety of marine mammals (Tanabe et al., 1984; Fiedler and Lau, 1998). For example, stable levels of PCBs in minke whale (*Balaenoptera acutorostrata*) blubber from 1984 to 1993 with a concurrent

decline in DDT suggest a continuous discharge of PCBs (Aono et al., 1997). Persistent levels of PCBs until the late 1980's also have been documented in northern fur seals and striped dolphins (*Stenella coeruleoalba*) (Loganathan et al., 1990; Tanabe et al., 1994). PCB levels in ringed seals from the Northwest Territories of Canada declined from 1972 to 1981 but remained constant until at least 1991 while DDTs continued to decline (Addison and Smith, 1998).

OC contaminant exposure elicits a broad spectrum of biological effects in animals (Tanabe et al., 1994; Safe, 1994). High doses are acutely toxic and chronic exposure to low levels currently found in the environment can induce adverse effects including immunosuppression in mammals and birds (Vos, 1977; Safe, 1984). High PCB levels in tissues are linked to low recruitment and reproductive dysfunction for the beluga (*Delphinapterus leucas*) population of the St. Lawrence River (Martineau et al., 1987). Additional residue studies have speculated on links between high OC residues and reproductive failure in a number of pinniped species (DeLong et al., 1973; Gilmartin et al., 1976; Helle et al., 1976a; Helle et al., 1976b; Reijnders, 1980; Addison, 1989; Baker, 1989; Zakharov and Yablokov, 1990; Bergman et al., 1992). Other studies have identified various OC-mediated toxic effects in harbor seals including reproductive impairment and immune dysfunction (Reijnders, 1986; De Swart et al., 1994; Ross et al., 1995; De Swart et al., 1996).

Low-level exposure to contaminants may cause death indirectly through increasing susceptibility to opportunistic pathogens. Contaminant-induced immunosuppression has been speculated to be a contributing factor to the extraordinarily

high mortality experienced by several marine mammal species during recent morbillivirus epizootics (Eis, 1989; Hall et al., 1992; Simmonds et al., 1993; Aguilar and Borrell, 1994a). Similarly, this mechanism is suggested to explain the high incidence, severity and diversity of lesions caused by opportunistic and mildly pathogenic bacteria found at necropsy in beluga from the St. Lawrence River estuary (Martineau et al., 1988; De Guise et al., 1995).

Although the immunotoxic effects of various environmental contaminants are well characterized in laboratory rodents and primates, quantifiable demonstration of these same effects in marine mammals is a major challenge. This is because of the paucity of baseline knowledge of the immune system in marine mammals and the scant availability of species specific reagents and limited validated assay development. Genetic diversity as well as logistical and ethical constraints are additional obstacles to demonstration of a cause-effect link even in captive research. However, a decrease in cellular and humoral immune responses in connection with chronic dietary contaminant exposure was documented by a controlled study in captive juvenile harbor seals fed wild-caught OC contaminated fish (De Swart et al., 1994; Ross et al., 1995; De Swart et al., 1995a; Ross et al., 1996). In addition, Lahvis et al. (1995) showed a negative correlation between blood PCB levels and T-lymphocyte responses to mitogen stimulation in 5 free-ranging bottlenose dolphins (*Tursiops truncatus*). From the evidence presently available, it appears that persistent environmental contaminants may represent a definite risk to the health of free-ranging marine mammals both in North America and in Europe (Ross et

al., 1996). However, a higher level of understanding is required for an assessment of the role of contaminants on free-ranging marine mammals.

Suckling is the major route of exposure to high concentrations of lipophilic organochlorine contaminants for mammalian neonates, as well as the major route of elimination of these chemicals for the mother through lactation (Addison and Brodie, 1977; Kodama and Ota, 1980; Wagemann and Muir, 1984; Yakushiji, 1988; Gallenberg and Vodicnik, 1989; Addison and Stobo, 1993; Borrell et al., 1995; Vrecl et al., 1996). While many marine mammal studies have concluded that transplacental transfer of OCs in marine mammals is low (Gaskin et al., 1971; Jones et al., 1976; Duinker and Hillebrand, 1979; Tanabe et al., 1982; Ronald et al., 1984; Martineau et al., 1987; Borrell et al., 1995), others concluded that transplacental transport is a significant source of OC exposure for offspring (Jones et al., 1976; Duinker and Hillebrand, 1979; Donkin et al., 1981; Born et al., 1981; Mössner et al., 1994).

The concentration of PCB congeners detected in milk of individual females is dependent upon the combination of recent dietary intake (prey selection), current body burden, metabolism, partitioning among tissue compartments (including blubber and blood) and secretion with milk lipids, and previous parity/lactation history (Addison and Brodie, 1977; Reijnders, 1980; Aguilar, 1983; Tanabe et al., 1986; Addison and Brodie, 1987; Addison, 1989; Aguilar and Borrell, 1994b; Ridgeway and Reddy, 1995; Espeland et al., 1997). Congener specific OC concentrations in a milk sample may be used to estimate exposure to the offspring in gray seals (*Halichoerus grypus)* (Pomeroy et al., 1996). In pinnipeds, the representation of individual PCB congeners in the milk is

different from the blubber (Addison and Brodie, 1987; von Schweigert and Knoppler, 1990). In a study of PCBs, polychlorinated dibenzo-*p*-dioxins and furans in blubber and milk samples from dam-pup gray seal pairs, Addison et al. (1999) concluded that "the transfer efficiency of CB congeners between maternal and pup blubber lipid was inversely correlated with lipid solubility and this selectivity of transfer appeared to occur partly at the stage of digestion of the milk by the pup". Comparisons of maternal blubber to pup blubber on a congener specific basis indicates selectively of transfer of PCBs with a trend for declining transfer efficiency with increasing chlorination occurring in a number of marine mammals including Dall's porpoise (*Phocoenoides dalli)* and harp (*Phoca groenlandica),* hooded (*Cystophora cristata*), and gray seals (Subramanian et al., 1988; Espeland et al., 1997; Addison et al., 1999).

First-born offspring of pilot whales (*Globicephalus melas*), fin whales (*Balaenoptera physalus*) and Steller sea lions (*Eumetopias jubatus*) are exposed to higher concentrations of lipophilic contaminants in milk than are subsequent offspring (Aguilar and Borrell, 1994b; Borrell et al., 1995; Lee et al., 1996). Lactational transfer of OCs to successive offspring is widely cited for decreasing OC adipose tissue levels with increasing age in adult females whereas levels increase with age in adult male pinnipeds and cetaceans (Gaskin et al., 1971; Addison and Smith, 1974; Donkin et al., 1981; Born et al., 1981; Tanabe et al., 1986; Martineau et al., 1987; Tanabe et al., 1987; Aguilar and Borrell, 1988; Aguilar and Borrell, 1994b; Borrell et al., 1995; Lee et al., 1996). Blubber from female northern fur seals off the coast of Japan in 1986 contained increasing PCB and DDT concentrations up to about 6 years of age, then decreased until age 20 (probably

the onset of senescence) (Tanabe et al., 1994). Conversely, in sperm whales (*Physeter macrocephalus)*, adult females tend to have higher contaminant burdens because they feed nearer to the surface than males and at lower latitudes (Aguilar, 1983). In fin whales, OC concentrations in males continue to increase after maturity while concentrations decrease non-linearly in females and tend to reach a plateau (Aguilar and Borrell, 1988). In growing animals, tissue OC concentrations increase as intake exceeds the animal's capacity for excretion but this also induces microsomal enzymes and increases the ability to metabolize the contaminants leading to the leveling off of age-related trends (Aguilar, 1987). In addition, different congeners show dissimilar trends across sexes associated with the differential action of microsomal enzymes and differential transfer rates of congeners in females (Aguilar and Borrell, 1988). Higher chlorinated PCBs increase with age because they are more difficult to degrade and therefore have a higher persistence than PCBs of lower chlorination (Aguilar and Borrell, 1988). Metabolic capabilities (i.e. P450-dependent monooxygenase system) vary significantly among marine mammal species as well as by age or sex (Duinker et al., 1989; Boon et al., 1992; Boon et al., 1997). The DDT/PCB ratio in fin whale showed a decreasing trend in females with age while increasing in males (Aguilar and Borrell, 1988). Thus, reproductive transfer in fin whales seemed to favor DDT compounds at a higher rate than the more lipophilic PCBs (Aguilar, 1987).

Free-ranging marine mammals are vulnerable to long term PCB toxicosis (Tanabe, 1988). As a result of their high trophic level, some species of marine mammals accumulate high levels of OC compounds, especially in lipid rich tissues such as blubber

(Bowes and Jonkel, 1975; Reijnders, 1980; Tanabe et al., 1981; Tanabe et al., 1984; Tanabe and Tatsukawa, 1991; Norstrom and Muir, 1994; Tanabe et al., 1994). Even with extraordinary high tissue burdens of contaminants, it is difficult to prove specific lesions are contaminant-induced in free-ranging marine mammals without captive studies to demonstrate direct cause and effect. Still, there a number of published examples of lesions in free-ranging marine mammals that are proximately linked to high tissue burdens of contaminants including: uterine stenosis in gray and ringed seals (Helle, 1980; Bergman and Olsson, 1985; Baker, 1989), adrenal hyperplasia in gray seals and beluga (Bergman and Olsson, 1985; Lair et al., 1997), a high prevalence of neoplasia in a beluga population (De Guise et al., 1994), hermaphrodite beluga and pseudohemaphrodite polar bears (*Ursus maritmus*) (De Guise et al., 1994; Wiig et al., 1998), skull lesions in gray and harbor seals (Zakharov and Yablokov, 1990; Bergman et al., 1992; Mortensen et al., 1992), and skin lesions in gray, ringed and northern elephant *(Mirounga angustirostris*) seals and sperm whales (Bergman and Olsson, 1985; Beckmen et al., 1997; Jauniaux et al., 1998).

Field study evidence is accumulating and based on the current information, organochlorine contaminants have the potential to significantly impact free-ranging pinniped populations (Hutchinson and Simmonds, 1994).

Scope of Study

The aim of this dissertation is to demonstrate the utility of an assessment of health status and immune functional capabilities of free-ranging northern fur seal pups.

As part of this assessment, the potential adverse impact of organochlorine contaminant exposure to pups via milk ingestion on health is investigated.

Chapter 2, " Factors Affecting Organochlorine Contaminant Concentrations in Milk and Blood of Northern Fur Seal (*Callorhinus ursinus*) Dams and Pups from St. George Island, Alaska", investigates the effect of dam age/parity on milk and blood levels of organochlorines in dam/pup pairs of fur seals captured during the periparturient period. This chapter demonstrates that northern fur seal pups of primiparous dams consume milk with higher concentrations of OCs than pups of multiparous dams early in the nursing period. This higher exposure from primiparous milk ingestion is subsequently reflected in the significantly higher OC concentrations in the blood of first-born pups. The influence of the year of capture and the percent lipid content on contaminant levels in blood and milk are considered. Biomagnification factors of PCB congeners and DDT metabolites from milk to pup blood and dam blood to pup blood are determined.

Chapter 3, " Age-dependent Effects of Organochlorine Contaminant Exposure on Hematological and Immune Functional Assays in Free-Ranging Northern Fur Seal Pups (*Callorhinus ursinus*)", explores the potential consequences of OC exposure on the health and immune function in fur seal pups using assessments of both humoral and cell-mediated immunity as well as standard health assessment techniques. Here, I address the hypothesis that pups with higher exposure to OCs and levels in whole blood would demonstrate hematological or immunological differences compared to pups with lower exposure either as neonates or one to two months later in the nursing period.

Cryopreservation of peripheral blood lymphocytes was tested and proved feasible under field conditions. This allowed use of cellular immune functional assays as part of the assessment, a technique not readily available when working with free-ranging marine mammals inhabiting such a remote site. Stimulation of the humoral immunity and examination of a number of blood indices in a recapture study made it possible to investigate differences in these indices correlated to pup age, dam age, OC exposure, and sex.

In Chapter 4, "Association of Organochlorines in Milk and Pup Blood with Serum Retinol and Thyroxine Levels in Northern Fur Seal Pups (*Callorhinus ursinus*)", the potential for neonatal organochlorine exposure to decrease serum retinol and thyroid hormone levels is investigated. The effect of age and sex of the pup on serum retinol and thyroid hormone levels is evaluated. Correlation coefficients between retinol, thyroid hormones and PCB congener concentrations are determined.

Chapter 5, "Effects of Parity and Dam Mass on Pup mass, Length, Growth, and Condition in a Cohort of Northern Fur Seal Pups (*Callorhinus ursinus*)" investigates whether a cohort of pups born to young, primiparous dams are born smaller, grow slower, and have poorer survival to 2 months-of-age than pups born to older, multiparous dams.

Table 1.1. Summary of ΣPCB concentrations in blubber of northern fur seals from the Pribilof Islands or Japanese waters by year of collection, age, and sex. Mean (± SD, when available) expressed as ng/g wet weight basis unless indicated as lipid wt. (l.w.). Values presented as orignally reported.

Age group	Sex (n)	Mean ± SD ng/g	Year of collection	Reference
Adult	F (2)	5800 ± 1500	1972	Kurtz and Kim, 1976 as Aroclor 1254
Adult	F (5)	4000 l.w.	1971-88*	Tanabe at al., 1994
Adult	na (1)	2320	not reported	Mössner and Ballschmiter, 1997
Subadult 2-5 y	M (4)	2490 ± 1060	1980	Calambokidis and Peard, 1985
Subadult 2-3 y	M (2)	103.8	1987	Schantz et al., 1993 as sum of 6 congeners
Subadult 2-5 y	M (7)	1343 ± 52	1990	Krahn et al., 1997 as sum of 17 congeners
Subadult 2-5 y	M (2)	1300 ± 570	90's	Varanasi et al., 1992
Subadult 2-5 y	M (2)	432	90's	Becker et al., 1997 as sum of 33 congeners
Subadult 2-3 y	M (10)	2293 ± 1045	1995	Beckmen, unpublished data
Pup, 2 mo.	na (3)	33000	1972	Kurtz and Kim, 1976 as Aroclor 1254
Pup, 1 mo.	na (4)	919 ± 332	1995	Beckmen, unpublished data
Pup, newborn	na (2)	5500	1972	Kurtz and Kim, 1976 as Aroclor 1254
Pup, stillborn	na (1)	396 l.w.	1990	Mössner et al., 1994 as sum of 7 congeners

na indicates sex not reported, * indicates from waters near Japan instead of Pribilof Islands

Table 1.2. Summary of ΣDDT concentrations in blubber of northern fur seals from the Pribilof Islands or Japanese waters by year of collection, age, and sex. Mean (± SD, when available) expressed as ng/g wet weight basis unless indicated as lipid wt. (l.w.). Values presented as originally reported.

Age group	Sex (n)	Mean ± SD ng/g	Year of collection	Reference
Adult	F (2)	5200 ± 2600	1972	Kurtz and Kim, 1976
Adult	F (5)	1700 l.w.	1971-88*	Tanabe at al., 1994
Subadult	M (1)	12700	1980	Calambokidis and Peard, 1985
Subadult 2-5 y	M (7)	2711 ± 1470	1990	Krahn et al., 1997
Subadult 2-5 y	M (2)	1280	90's	Becker et al., 1997
Subadult 2-5 y	M (2)	1800 ± 800	90's	Varanasi et al., 1992
Subadult 2-3 y	M (10)	3617 ± 2137	1995	Beckmen, unpublished data
Pup, 4 mo.	na (5)	15800	1969	Anas and Wilson, 1970
Pup, 2 mo.	na (3)	63000	1972	Kurtz and Kim, 1976
Pup, 1 mo.	na (4)	1440 ± 891	1995	Beckmen, unpublished data
Pup, newborn	na (2)	5600	1972	Kurtz and Kim, 1976
Pup, stillborn	na (1)	2965 l.w.	1990	Mössner et al., 1994

na indicates sex not reported, * indicates from waters near Japan instead of Pribilof Islands

Chapter 2

Factors Affecting Organochlorine Contaminant Concentrations in Milk and Blood of Northern Fur Seal (*Callorhinus ursinus*) Dams and Pups from St. George Island, Alaska[1]

2.0 Introduction

Breeding rookeries for more than 72% of the world's population of northern fur seals (*Callorhinus ursinus*) are located in the Bering Sea on the two largest Pribilof Islands, St. Paul and St. George, Alaska (Loughlin et al., 1994). Pups are born from late June to early August. After four months of nursing, the pups spontaneously wean and migrate in November (Gentry, 1998). During the winter, pregnant fur seals and juveniles of both sexes feed in wide areas of the North Pacific including continental shelf areas off North America, Russia and Japan (Bigg, 1990).

The number of pups born (pup production) on St. Paul Island has stabilized following a decline of about 7% per year from 1975-1983 (York, 1990). However, on St. George Island, pup production declined approximately 4.7% per year from 1970 until 1994 (York et al., 1997). The latest pup production estimates for St. George Island showed an increase in production from 1994 but further assessment should be conducted to reveal any trend (York et al., 1997). The current Pribilof stock abundance is less than

[1] Beckmen KB, Ylitalo GM, Towell RG, Krahn MM, O'Hara TM, Blake JE.1999. Factors affecting organochlorine contaminant concentrations in milk and blood of northern fur seal (*Callorhinus ursinus*) dams and pups from St. George Island, Alaska. The Science of the Total Environment (In Press).

half of historical levels and is listed as depleted under the Marine Mammal Protection Act (Loughlin et al., 1994; York et al., 1997). Poor pup production reflected a population decline. This decline was attributed mainly to reduced recruitment due to poor juvenile survival during the 20 month post-weaning migration and some increase in adult female mortality at sea (Trites and Larkin, 1989; York, 1990; Trites, 1992).

A contributing factor to poor juvenile survival may include exposure to organochlorine (OC) environmental contaminants such as polychlorinated biphenyls (PCBs) and dichloro-diphenyl-trichloroethane (DDTs), which have been detected in fur seal milk and pup tissues (Anas and Wilson, 1970a; Anas and Wilson, 1970b; Kurtz and Kim, 1976; Bacon et al., 1992; National Marine Fisheries Service, 1993; Mössner et al., 1994). The effects of OC exposure on pup health or reproduction have not been previously investigated. Most contaminant surveys have focused on tissue residues in subadult males readily obtained from the Alaska native subsistence harvest. Although information is valuable, it provides no insight into an impact assessment (Calambokidis and Peard, 1985; Calambokidis, 1987; Varansi et al., 1992; Schantz et al., 1993; Becker et al., 1997; Krahn et al., 1997). High PCB levels in tissues are linked to low recruitment and reproductive dysfunction for the St. Lawrence beluga (*Delphinapterus leucas*) population (Martineau et al., 1987). Additional studies have speculated that high OC residues and reproductive failure may be linked in a number of pinniped species (DeLong et al., 1973; Gilmartin et al., 1976; Helle et al., 1976a; Helle et al., 1976b; Reijnders, 1980; Addison, 1989; Baker, 1989; Zakharov and Yablokov, 1990; Bergman et al., 1992). Other studies have identified various OC-mediated toxic effects in harbor seals

(*Phoca vitulina*) including reproductive impairment and immune dysfunction (Reijnders, 1986; De Swart et al., 1994; Ross et al., 1995; De Swart et al., 1996).

Suckling is the major route of exposure to high concentrations of lipophilic OC contaminants for mammalian neonates, as well as the major route of elimination of these chemicals for the dam through lactation (Kodama and Ota, 1980; Wagemann and Muir, 1984; Yakushiji, 1988; Gallenberg and Vodicnik, 1989; Addison and Stobo, 1993; Vrecl et al., 1996). Studies have generally concluded that transplacental transfer of OCs in marine mammals is low (Gaskin et al., 1971; Jones et al., 1976; Duinker and Hillebrand, 1979; Tanabe et al., 1982; Martineau et al., 1987). In marine mammals, concentrations of PCB congeners detected in milk of individual females are dependent upon the combination of recent dietary intake (prey selection), current body burden, metabolism, partitioning among tissue compartments (including blubber and blood), secretion with milk lipids, and previous parity/lactation history (Addison and Brodie, 1977; Reijnders, 1980; Tanabe et al., 1986; Addison and Brodie, 1987; Addison, 1989; Aguilar and Borrell, 1994b; Ridgeway and Reddy, 1995).

First-born offspring of humans, pilot whales (*Globicephalus melas*) and fin whales (*Balaenoptera physalus*) are exposed to higher concentrations of lipophilic contaminants in milk than are subsequent offspring (Rogan et al., 1986; Yakushiji, 1988; Aguilar and Borrell, 1994b; Borrell et al., 1995; Vartiainen et al., 1997). For example, 80% of the OC burden in a lactating Steller sea lion (*Eumetopias jubatus*) may be transferred to her first offspring (Lee et al., 1996). Lactational transfer of OCs to successive offspring is widely cited for decreasing OC adipose tissue levels with

increasing age in adult female pinnipeds and cetaceans whereas levels increase with age in adult males. Blubber from female northern fur seals off the coast of Japan in 1986 contained increasing PCB and DDT concentrations up to about 6 years of age, then decreased drastically until age 20, probably the onset of senescence (Tanabe et al., 1994).

Suckling neonates are at critical stages of neurological and immune system development and are particularly sensitive to adverse effects of OCs, even at low level exposure (Carstens et al., 1979; Thomas and Hinsdill, 1980; Bleavins et al., 1984; Thomas and Faith, 1985; Huisman et al., 1995; Pluim et al., 1996). Transfer of high concentrations of lipophilic organochlorine compounds via lipid-rich fur seal milk could have deleterious effects on the early critical development of fur seal pups. Potential effects include immune function disorders leading to increased susceptibility to infection and increased indirect mortality post-weaning. Variation in the sensitivity to toxic effects is dependent not only on developmental age, but also the gender, species, dose, route of exposure and congener make-up (McConnell, 1985; Tilson et al., 1990; Safe, 1994).

In this report, blood concentrations of select PCB congeners and pesticides from northern fur seal pups born to primiparous vs. multiparous dams are compared. Additionally, maternal blood and milk contaminant levels are compared between these two groups. The influence of the year of capture and the percent lipid content on contaminant levels in blood and milk are considered. Biomagnification factors of PCB congeners and DDT metabolites from milk to pup blood and dam blood to pup blood are determined.

2.1 Methods

Study site

Northern fur seals were captured on four of the six rookery beaches of St. George Island, Alaska (N56° 34' W169° 41'). This is one of the two main breeding islands in the Pribilof group where northern fur seals gather between May and October each year to reproduce.

Animal criteria and capture techniques

Northern fur seal "neonates" and dams were captured between 17 July – 5 August 1995 and 13 July – 3 August 1996, with a noose pole extended from a 3-person roving 'blind box' constructed of plywood (approximately 1x1.5x2m) using methods detailed elsewhere (Antonelis, 1992; Boltnev et al., 1998). This 'blind box' afforded protection of personnel from aggressive territorial bulls and allowed access to most of the rookery with minimal animal disturbance.

All neonates were accompanied by their dams at the time of initial capture and dams were classified as young (≤5 years of age and likely to be primiparous) or old (>7 years, assumed to be multiparous) by body size, pelage and vibrissae characteristics as described by Vladimirov and Nikulin (1993). The vast majority of neonates included in the study were estimated to be less than 7 days old, based on the condition of the umbilical cord remnant or the degree of umbilical healing, and neonatal behavior and observation of characteristic periparturient/pre-estrus behavior of the dam (Gentry, 1998). The pups were observed in contact with their dams when selected for capture, but the timing, duration, and volume of their most recent suckling bout was not known.

Sample collection

Seals were physically restrained for sample collection. Blood samples were obtained from one of the superficial plantar flipper veins using a 21 gauge Venoset® blood collection set into Venoject II® 5 ml plastic vials containing dry EDTA (Termo Medical Corp. Elkton MD). After gentle mixing, the anti-coagulated whole blood samples were transferred to methylene chloride-rinsed glass vials with Teflon lined lids. A milk sample of approximately 5 ml was obtained from each dam about 5 minutes after a 5 IU intramuscular injection of oxytocin (Vedco, Phoenix Scientific Inc., St. Joseph, MO). Milk was collected midstream by manual expression directly into similar vials. Milk and blood samples were frozen (-20 °C) within 6 hours of collection until thawed for analysis. Capture and sampling activities were conducted under Marine Mammal Protection Act/Fur Seal Act permits #837 and #1003 and a research protocol approved by the Institutional Animal Care and Use Committee of the University of Alaska Fairbanks.

Analytical techniques

Concentrations of select PCB congeners and other organochlorine compounds were determined using a rapid high-performance liquid chromatography (HPLC) coupled with photodiode array detection method as previously described (Krahn et al., 1994). Briefly, 3-4 g of whole blood or 0.5-0.9 g of milk were thawed and weighed to the nearest 0.01 g and homogenized with 20 ml of hexane/pentane (1:1 v/v), sodium sulfate and the surrogate standard (1,7,8-trichlorodibenzo-*para*-dioxin; 250 ng), centrifuged and decanted into a concentrator tube. The homogenization process was repeated on the

sample remaining in the centrifuge tube and the extracts were combined. A 1 ml portion of extract was removed for lipid quantitation. The remaining sample extract was evaporated down to a volume of approximately 1 ml. The sample extract was loaded onto a custom gravity-flow cleanup column (which contained a glass wool plug, silica gel, basic silica gel and acidic silica gel) to separate the CBs from other interfering compounds (i.e. lipids, aromatic hydrocarbons). The CBs were eluted from the cleanup column with 14 ml of hexane/methylene chloride (1:1 v/v) and collected into a concentrator tube. The HPLC internal standard was added to each sample (1,2,3,4-tetrachlorodibenzo-*para*-dioxin; 250 ng) and the solvent volume was reduced to approximately 150 µl under nitrogen evaporation. Eleven dioxin-like congeners (CBs -77, -81, -105, -118, -126, -156, -157, -169, -170, -180 and -189) were resolved from other selected CBs (CBs -101, -128, -138, and -153), hexachlorobenzene (HCB) and chlorinated hydrocarbons (e.g. *p,p*'-DDD, *p,p*'-DDE, *p,p'*-DDT) by HPLC on 2 (1-pyrenyl) ethyldimethylsilylated silica (PYE) analytical columns (connected in series) cooled to 9°C and were detected with a Waters 996 PDA detector (Waters, Milford, MA). These analytes were identified by comparing their UV spectra (200-310 nm) and retention times to those of reference standards in the PDA library (Krahn et al., 1994). Compound purity was confirmed by comparing UV spectra collected for a peak to the apex spectrum. In this method there is co-elution of the minor CBs -99/149/196 with possibly others and CB-101; and CB-87 with CB-153; and CB-194 with CB-170 (Krahn et al., 1994). The lower limits of detection for congeners in milk were 0.04 ng/g and 0.05 ng/g for blood.

Total lipid concentrations of blood and milk samples were determined by thin layer chromatography with flame ionization detection (TLC/FID) using an Iatroscan Mark 5 (Iatron Laboratories, Tokyo, Japan). The extracts were spotted on Chromarods (Type SIII) and developed in a solvent system containing 60:10:0.02 hexane:diethyl ether:formic acid (v/v/v). Various classes of lipids (i.e., wax esters, triglycerides, free fatty acids, cholesterol, and phospholipids) were separated based on polarity, with the nonpolar compounds (i.e., wax esters) eluting first followed by the more polar lipids (i.e., phospholipids). The Iatroscan was operated with a hydrogen flow rate of 160 ml/min and air flow of 2000 ml/min. Data was acquired and analyzed using TDatascan software (RSS Inc., Bemis, TN). A four-point linear external calibration was used for quantitation. Total lipid concentrations were calculated by adding the concentrations of the five lipid classes for each sample and were reported as percent total lipid. Duplicate TLC/FID analyses were performed for each sample extract and the mean value recorded.

Calculated values

Using the areas under the congener curves and concentrations determined from the HPLC/PDA chromatograms, the total PCB concentrations were calculated by the following formula:

Total PCBs = sum of concentrations of selected CB + sum of concentrations of other CBs. Individual analyte and total PCB concentrations were reported as ng/g wet weight. The TCDD (tetrachlorodibenzo-*para*-dioxin), or dioxin-like, toxic equivalency quotients (TCDD TEQs) were calculated using an additive model of toxicity (Safe, 1990). In this method, molar concentrations of the dioxin-like congeners were multiplied

by their toxic equivalency factors (TEFs) recently recommended by the World Health Organization-European Centre for Environment and Health (WHO-ECEH) and the International Programme on Chemical Safety (IPCS) (Ahlborg et al., 1994). When the concentration of a dioxin-like CB was below the limit of detection, the TCDD TEQ value for that specific congener was not used in the calculation. The TCDD TEQs were reported as pg/g wet weight. The ratio of ΣDDTs to total PCBs (ΣDDTs/PCBs) was calculated on a wet weight basis for each animal by a simple ratio of the sum of the DDT metabolites over the total PCBs. The mean and standard deviation for the entire group is reported.

Data were normalized for comparison across matrices in two ways. First, lipid-adjusted (l.w.) concentrations were calculated on the basis of the percent lipid content of the individual blood or milk sample: (concentration of CB_x in ng/g w.w.) / (percent lipid of sample / 100) . A ratio of the wet weight concentrations of each congener [CB_x] to the concentration of the recalcitrant (non-metabolized) congener CB-153 was calculated and reported as R_{153} (CB_x) which equals [CB_x] / [CB_{153}]. These ratios were subsequently used to determine the relative ratio of each $R_{153(CBx)}$ normalized congener from a dam's milk to her pup's blood derived from the calculation:

$$Rrel_{(CBx)} = R_{153\ (CBx)}\ \text{Pup blood} / R_{153\ (CBx)}\ \text{milk}.$$

This depicts the relative biomagnification factor from prey/food source to the consumer (Boon et al., 1994) and was adapted here using the milk as a food source (prey) and the pup's blood as the consumer. A significantly elevated Rrel >1.0 (i.e. 95%

confidence interval lower limit is >1) indicates a significant biomagnification from one trophic level to the next (in this case from milk to pup blood).

The minimum "biomagnification factor" (BMF) was calculated as a simple ratio of each pup blood congener concentration (l.w.) divided by the corresponding maternal milk or blood concentration. If the dam blood sample was below the detection limit for a congener when the pup blood had measurable levels of the congener, then the detection limit was used for the denominator, thus the BMF reported is the minimum value. When the minimum value of the 95% confidence interval is greater than 1.0 for a BMF, biomagnification is considered significant.

PCB congener and pesticide metabolite concentrations were compared on a wet weight basis (w.w.) and lipid weight basis (l.w.) as noted. Comparisons are made for each matrix by year of collection (1995 and 1996). Concentrations in each matrix were grouped according to the relative age of the dam (young, presumably primiparous versus old, presumably multiparous) and compared to detect significant differences. Comparisons were made between the milk and blood OC levels of young dams and old dams. Pup blood OC levels grouped by their dam's age were compared as well. Differences in concentrations between groups were determined by Student's *t*-tests when distribution was normal and variances were equal, otherwise a Mann-Whitney Rank Sum Test was used with a significance level of $p < 0.05$ (exceptions will be noted). Statistical analyses were performed using Sigma Stat 2.0® (SPSS, Chicago, IL). Results are reported as mean and standard deviation (SD) in tables and are represented as mean and standard error (SE) in figures except where noted.

2.2 Results

PCB congener and DDT metabolite concentrations by matrix

In 1995, 11 dams (6 young, 5 old) were sampled along with their pups. In 1996, 22 dams (11 young, 11 old) were sampled along with their pups, plus additional 27 pups (with dams in attendance). Toxicological analyses were conducted on 33 milk samples, 59 pup blood samples and 29 dam blood samples.

CB-77, -81, -126, -169, and -189 were below the detection limits (BDL) of the assay (approximately 0.04 ng/g) in all samples. Congeners CB-156 and -157 were not detected in blood but were detected in some milk samples (25 and 5 of 33, respectively). Hexachlorobenzene (HCB) was only detected in two milk samples and is not included in the data analysis. A statistically significant difference between year of sampling (1995 and 1996) for some PCB congeners (Fig. 2.1) and DDT metabolite levels (data not shown) for both milk and blood on a wet weight and lipid weight basis was detected. There were notable differences in milk PCB congeners CB-101/99/149/196, -128, -156, and -157. Independent analysis of the data by year of collection was required to remove this confounding variable for subsequent statistical comparisons. Only 1996 samples will be presented in the subsequent tables and figures for significant differences between means because of the larger sample size collected in that year.

Tables 2.1 and 2.2 summarize the mean and SD (w.w.) of specific congeners and DDT metabolites, respectively, in each matrix of the dams and pups from 1996. The mean ± SD toxic equivalency quotients of dioxin-like congeners (TCDD TEQs) were 7.1

± 3.8 pg/g for milk, 0.09 ± 0.09 pg/g for pup blood and 0.03 ± 0.03 pg/g for dam blood at sample sizes of 22, 19 and 48, respectively.

OC concentrations in pup blood vs. milk

The concentrations of CB-101, -105, -118, -138, -153/87, 170/194, -180, and total PCBs were significantly higher in 48 pup blood samples (Mann-Whitney Rank Sum Tests at $p < 0.001$) compared to the lipid-normalized concentrations of each congener from 22 milk samples in 1996. Mean + SE concentrations in milk, pup and dam blood are presented in Figure 2.2.

When comparing 21 paired samples of pup blood to their respective dam's milk (l.w.), CB-153/87, CB-138, and CB-118 concentrations were higher in 85 % of pup's blood samples (by factors up to 7, 8, and 10, respectively), and CB-101/99/149/196 concentration was higher in 77 % of the pups (by a factor as high as 19).

Comparison of pup blood vs. dam blood OC concentrations

There was no significant difference in mean blood percent lipid between dam and pup samples (0.2 ± 0.1 versus 0.3 ± 0.3) allowing a comparison based on wet weight concentrations. Wet weight median concentrations of CB-101, -118, -138, -153/87, and total PCBs were all significantly higher (Mann Whitney Rank Sum Test $p < 0.001$) in the blood of pups than dams (Table 2.1). TCDD TEQs were also significantly higher in pup blood than dam blood (0.09 ± 0.09 pg/g versus 0.03 ± 0.03 pg/g). CB-153 and CB-180 were also significantly greater on a lipid weight basis in pup blood (Figure 2.2). CB-128 and -170/194 were detected in four and three pup blood samples, respectively, but were below the detection level in all dam blood samples.

Mean *p,p'*-DDE and ΣDDT levels followed the same trend of significantly higher concentrations in pup blood than dam blood (w.w.) (Table 2.2). Pup blood concentrations of *p,p'*-DDE ranged from 0.58 to 40 ng/g (w. w.) whereas dam blood ranged only from 0.06 to 3.3 ng/g. Of the lesser DDT metabolites, *p,p'*-DDT and *p,p'*-DDD were detected in five and three pup blood samples, respectively, but were below the detection level in all dam blood samples. *o',p'*-DDD was not detected in any of the blood samples.

Comparison of dam blood and milk

Only the lipid-normalized concentration of CB-101/99/149/196 was significantly higher in the blood of dams than in the milk samples (mean 384.3 ± 187.0 versus 217.8 ± 133.6 ng/g, $p < 0.02$). CB-128, -156, -157, -170/194, *p,p'*-DDT, *o,p'*-DDT, *p,p'*-DDD and *o,p'*-DDD were not detected in dam blood samples, but were detected in milk samples. CB-180 was detected in blood samples of only two dams, but both were higher (l.w.) than in the respective milk sample. Conversely, *p,p'*-DDE was greater in milk than in the dam's blood (w.w. and l.w.). The ΣDDTs/PCBs were significantly higher in milk than in the dam's blood (Table 2.2).

PCB congener concentrations by dam age

PCB concentrations in 48 pups and 22 dams were compared based on the relative dam age categories, young or old. The percent lipid in milk from young dams was significantly lower (22.7 ± 6.4) than percent lipid in the milk from older dams (29.7 ± 6.5) (Students-*t* test at $p < 0.05$) therefore the milk concentrations were lipid-normalized for comparisons. The levels of CB-101, -128, -138, -153/87, -170/194, and -180 were

significantly higher in the early lactation milk of young dams compared to old dams (l.w.) (Figure 2.3). CB-156 and -157 were frequently below the limits of detection in milk and thus did not allow for statistical comparison of concentrations between age groups. However, CB-156 and -157 were detected in 9 and 3 of the 11 young dam's milk samples, respectively, but only in 4 and 1 of the 11 old dam's milk samples, respectively.

Figure 2.4 depicts significant differences in mean blood concentrations (w.w.) in both pups and dams categorized by the relative age category of the dams. In blood, CB-105 and -180 were detected only in young dams and pups; CB-128 and -170/194 were below detection limits in all dam blood samples, but were detected in pups of young dams only. The mean concentrations of seven of eight congeners were significantly greater (w.w.) in pups of young dams compared to pups of old dams (Figure 2.4). This trend held for TCDD TEQs (Figure 2.5) and total PCBs. The mean concentrations of *p,p*'-DDE (w.w. and l.w.) in the blood of pups of young dams were higher than in the blood of pups of old dams (Figure 2.6). Pups of young dams also had greater mean concentrations of *p,p'*-DDE in blood than dams regardless of age. The mean concentration of *p,p'*-DDE in pups of old dams was not significantly different from the mean concentration in the blood of old dams.

"Biomagnification and bioaccumulation"- type model

The mean "biomagnification factor" (BMF) and the comparison of the 95% confidence interval to unity for eight congeners and three DDT metabolites are presented in Figure 2.7. Columns indicate the minimum mean BMF with a standard error bar as a ratio of pup blood (when above detection limit) divided by dam milk or blood (l.w.).

Thus, CBs-101, -105, -118, -138, -153/87, and -180 are “biomagnified” from the milk based on BMF’s >1.0. CBs-128, -170/194 and *p,p'*-DDT were only detected in 1, 2 and 3 respectively, of the 21 pups but in those cases the BMF was still greater than 1. The BMF from dam’s blood to her pup’s blood were similar except for CB-138, *p,p'*-DDE and CB-105 which are greater. The BMFs calculated through blood are conservative since some dams were below the detection limit for each congener while their pup had detectable levels except CB-153/87 and *p,p'*-DDE where all blood samples had measurable levels.

Figure 2.8 plots the R_{rel} congeners from a dam’s milk to her pup’s blood. Based on R_{153} normalized data, indications are that CB-101/99/149/196 and CB-170/194 are bioaccumulated in the pup's blood (R_{rel} >1.0) relative to dam milk but only two pup blood samples were above the detection level for CB-170/194. R_{rel} (105) was widely variable and R_{rel} (118) and R_{rel} (138) do not indicate bioaccumulation as it did based on the lipid normalized BMF. The reported means for R_{rel} (101/99/149/196) and R_{rel} (170/194) are >1 but only R_{rel} (170/194) has a 95% confidence lower limit >1 though it is based on very few samples above the detection limit.

2.3 Discussion

The results demonstrate that northern fur seal pups of primiparous dams consume milk with higher concentrations of OCs than pups of multiparous dams early in the nursing period. This higher exposure from primiparous milk ingestion is subsequently reflected in the significantly higher OC concentrations in the blood of first-born pups. Significant mean "biomagnification factors" ranging from 1.5 to 7.5 were also determined from milk to pup blood. Higher OC exposure in the first-born was consistent with studies in other mammals but the magnitude of the difference for neonatal pups is remarkable.

Shortly after birth, the OC concentrations in a pup's blood is higher than in either the dam's milk (l.w.) or blood (w.w.). This supports a previous study that concluded transplacental transfer occurred in this species and was quite significant in establishing the initial contaminant burden in the newborn (Mössner et al., 1994). This is also consistent with a 1972 study addressing OCs in northern fur seal dams and their newborn pups (Kurtz and Kim, 1976). They documented higher levels of PCBs and DDTs in the fat of one of two newborns than in their dams (blood PCB levels were below the detection limits of the method used). The combination of *in utero* and post-natal exposure to OC's is confounding, but important because of the levels observed at this critical developmental life stage.

Human infant fat OC levels increase rapidly in the first three months and eventually exceed the OC levels in the mother's fat (Mes et al., 1984). In humans, during breast-feeding, the PCB concentrations in milk and maternal blood fall 2-3 fold while

PCB concentration in the baby's blood increases 6-7 fold over a 2 year period. By the end of nursing, the baby's blood concentration of PCBs is 5-6 times higher than that of the mother (Kodama and Ota, 1980). In marine mammals, the transfer is more rapid. For example, a bottle-nosed dolphin (*Tursiops truncatus*) calf received 80% of its dam's PCB and ΣDDT burden in the first seven weeks post-partum (Cockcroft et al., 1989). In gray seals (*Halichoerus grypus*), pups receive 98% of their first year's body burden of OC in the 16-day nursing period (Addison and Stobo, 1993). Rodent studies also demonstrate that in the first few post-partum days, neonates receive a higher dose of OCs on a body weight basis than was originally administered to the dam (Gallenberg and Vodicnik, 1989). It appears that fur seal neonates may experience a similar rapid high dose transfer.

There were no significant differences between the extractable lipid contents in dam and pup blood but comparison of l.w. PCB concentrations to other studies is complicated by differences in extraction methods (Randall et al., 1998). Other studies have used a gravitational method that measures total extractable solids instead of chromatography of the extractable lipids. This is not a concern when comparing the blood sample concentrations of PCBs within this study.

Another factor that must be considered for the comparison between dam and pup blood on a w.w. basis is fasting versus non-fasting states (Matthews et al., 1984b). Pups in this study were likely to have recently suckled since their dams were all in attendance at capture but the time since foraging for the dams was not known. Again, significant differences in OC levels between dam and pup blood were found both on w.w. and l.w. concentrations so the differences were not solely due to the non-fasting state of pups.

The findings indicate that lipid-adjusted total PCB concentrations are higher in maternal blood than in milk. This is in contrast to human breast milk at birth where the concentration of PCBs are approximately equal to those of the maternal blood when lipid-adjusted (Masuda et al., 1978; Gobas et al., 1993). But fur seal milk has a significantly different lipid content than in humans (Ashworth et al., 1966; Bauman and Davis, 1974). Since PCBs are in dynamic equilibrium with the body stores and the blood, the onset of lipid secretion by lactation should result in the partitioning of PCBs from the blood into the milk and a corresponding movement of PCBs from all tissues into the blood in order to maintain their respective tissue/blood ratios (Matthews and Dedrick, 1984). However, each congener may have a unique temporal partitioning relationship, and the measured levels in blood and milk were from a single time point. Boon et al. (1994) found lipid normalized blood concentrations of PCB congeners in harbor seals were often higher by a factor of up to five compared to liver or blubber. This was explained by the adsorption of a significant portion of the PCBs to the non-lipid constituents, e.g. non-polar parts of the serum albumin.

The “biomagnification factors” or accumulative exposure levels reported here support selective partitioning (including absorption) of PCB congeners into northern fur seal milk. Investigators working with gray seals have found the net transfer of PCBs from maternal blubber to blood to milk is about 70% efficient with some selectivity for lower chlorinated PCBs from blood lipid to milk (Addison and Brodie, 1987). They also speculated on a physiological barrier in the mammary gland of gray seals that is more effective in restricting PCB passage than DDT into the milk (Addison and Brodie, 1977;

Addison and Brodie, 1987). They found that levels of DDTs and PCBs in milk lipid were approximately 60 and 30% of levels in maternal blubber lipid, respectively. They also suggest that there was selection against movement of the lower chlorinated PCB congeners from circulatory lipids into milk lipid compared with the DDTs (Addison and Brodie, 1987). Thus, they propose that a non-selective barrier exists to quantitative transfer of residues from blubber lipid to circulatory lipid, and a partially selective barrier based on lipid solubility exists that favors DDTs over PCBs in the transfer of residues from circulatory lipid to milk lipids. The increased lipid solubility (affinity for triglycerides and non-esterified fatty acids) of the more highly chlorinated PCBs and pesticides results in higher concentrations relative to lower chlorinated PCBs in the adipose tissue and milk compared to blood or serum (Masuda et al., 1979; Aguilar, 1985; Massé et al., 1986; Addison and Brodie, 1987; Kawai et al., 1988). Based on data from other species, a lactating northern fur seal likely mobilizes blubber triglycerides and transports them via blood to secrete in milk but synthesizes the majority of milk triglycerides *de novo* in the mammary gland (up to 80% in some species) (Bauman and Davis, 1974). It appears that OCs accumulated over the first 4-5 years of life until the first lactation period are partitioned out of blubber and into the milk, especially those congeners with higher chlorine substitutions. These congeners are then transferred to the first born pup. Human, ferret, and rodent studies confirm that lactational transfer of OCs in these species occurs mainly from fat residues and is largely unaffected by the diet during lactation (except during starvation) (Masuda et al., 1979; Bleavins et al., 1984; Jacobson et al., 1984; Skaare et al., 1988).

The results indicate biomagnification of PCBs in the blood of pups relative to the milk they ingest. CB-180 is typically resistant to biotransformation in most species and accumulation (BMF > 1.0) was as expected (Muir et al., 1988; 1992). The variation in the biomagnification from the milk to the pup suggests some selective secretion of these congeners in milk as discussed above or, alternatively, selective absorption across the gastrointestinal mucosa. By re-analyzing the data of Kodama and Ota (1980), Gobas et al. (1993) concluded that intestinal uptake of hydrophobic organic compounds in ingested milk occurs by passive diffusion, and some biomagnification occurs within the gastrointestinal tract via digestion. The subsequent partitioning of OCs into the blood and tissues of the neonate is therefore not strictly a consequence of a compound's lipophilicity.

Biomagnification factors have been used to describe the accumulation of a lipophilic contaminant from one trophic level to the next up the arctic marine food chain (Muir et al., 1988). In the present study, the adaptation of BMFs to estimate apparent magnification in the trophic transfer of mother to pup via the secretion and subsequent consumption of milk is on the same order as the trophic transfer from fish to ringed seal (*Phoca hispida*) (Muir et al., 1988). Therefore, from a contaminants perspective, the pup is essentially feeding at a higher trophic level than the dam. Significant differences in the induction and efficiencies of metabolic enzymes (e.g. cytochrome P-450) in the liver of the pup, as compared to those of the dam, must also be considered as factors. Induction of liver enzyme activities can occur from transplacental as well as transmammary exposure to OCs (Örberg, 1977; Vodicnik et al., 1980; Vodicnik and Lech, 1982;

Gallenberg and Vodicnik, 1989). The metabolic status of northern fur seal pups is currently unknown but under study.

The biomagnification of CB-101/99/149/196, -105, -118, -170/194, -180 in fur seal pups suggests that their metabolic capacity for various PCBs may be similar to cetaceans and unlike phocid seals (Tanabe, 1988; Boon et al., 1992). However, analyzing a select number of congeners hampers this metabolic assessment and very little data is available on northern fur seal metabolism of OCs. It has been speculated that immature (low activity) metabolic pathways and the dynamic alterations occurring in the rapidly developing neonate have a considerable effect on the manifestations of PCB toxicosis (Barsotti and Van Miller, 1984).

It was unexpected that significant differences in congener concentrations in blood and milk would be detected from 1995 to 1996. The short time scale makes it unlikely that a change occurred in the actual environmental contaminant levels. A long-term (decadal) decline in the concentrations of all OCs in milk was expected because of the discontinued manufacture and reduction in use of most of these compounds. However, this was not evident by comparing geometric means of 1996 milk samples to a previous study of seven fur seal milk samples collected on St. George Island in 1981 (Bacon et al., 1992). Caution must be exercised when interpreting comparisons across studies using different analytical methods. Yet, it appears there was an increase of 3.8% for the sum of the six PCB congeners common to both studies. There was a more dramatic change in the DDT metabolites, a 35% increase in reported geometric mean of ΣDDT from 1981 to 1996 (519 to 701 ng/g) with the geometric mean concentration of *p,p'*-DDE increasing

from 510 ± 15 to 610 ± 62 ng/g and the ratio of *p,p'*-DDT to *p,p'*-DDE increasing from ~1 to 5.3. No dam ages were given for the 1981 milk samples. They reported a substantially higher lipid content (geometric mean of 37% versus 25%), therefore, on a lipid-adjusted basis, the increase would be greater. Confounding factors introduced by analytical differences such as the co-elutions of other congeners must also be considered. Yet there is an indication that in the past 15 years, concentrations of these PCB congeners and DDT metabolites have not decreased in northern fur seal milk, conversely they have increased. Tanabe et al.(1994) found relatively steady concentrations of PCBs in blubber from female northern fur seals in the western Pacific from 1979 until 1988 after the peak level in 1976. They further suggest that the temporal patterns and congener compositions of PCBs in northern fur seals along with similar data from striped dolphins (*Stenella coeruleoalba*) published by Loganathan et al.(1990), indicate there were still significant continuous inputs of PCBs into the marine environment at least until the late 1980s. The PCB levels in northern fur seal milk lend support to the possibility that PCB inputs continued at least into the mid-1990s.

Table 2.1.Mean ± SD concentrations (number below detection) of PCB congeners in ng/g wet weight basis in northern fur seal blood and milk collected on St. George Island, Alaska, 1996.

	Milk n = 22	Dam Blood n = 19	Pup Blood n = 48
CB-101/99/149/196	50.0 ± 21.2	0.5 ± 0.2	1.1 ± 0.8*
CB-105	16.0 ± 11.7	0.3 ± 0.1 (17)	0.4 ± 0.2 (33)
CB-118	46.4 ± 22.3	0.4 ± 0.2 (4)	0.9 ± 0.6 (4)*
CB-128	8.0 ± 4.7	< 0.04[a]	0.3 ± 0.1 (44)
CB-138	42.3 ± 23.4	0.3 ± 0.1 (3)	0.9 ± 1.2 (2)*
CB-153/87	79.1 ± 44.6	0.4 ± 0.2	1.3 ± 1.1*
CB-156	2.7 ± 0.9 (8)	< 0.04[a]	< 0.04[a]
CB-157	1.4 ± 0.4 (17)	< 0.04[a]	< 0.04[a]
CB-170/194	5.3 ± 3.2 (1)	< 0.03[a]	0.4 ± 0.1 (45)
CB-180	13.6 ± 8.0	0.3 ± 0.1 (17)	0.4 ± 0.2 (25)
Total PCBs	433.4 ± 197.8	14.5 ± 2.6	20.6 ± 6.9*
%Lipid	26.2 ± 7.5	0.2 ± 0.1	0.3 ± 0.3

*pup blood level significantly greater than dam blood level, $p < 0.001$

[a]all samples below this detection limit

Table 2.2. Mean ± SD concentrations (number below detection) of DDT metabolites ng/g (w.w.) in blood and milk in northern fur seals on St. George Island, Alaska, 1996.

	Milk *n* = 22	Dam Blood *n* = 19	Pup Blood *n* = 48
p,p'- DDE	757.7 ± 450.7	1.4 ± 0.9	7.8 ± 9.2*
p,p'- DDT	35.3 ± 12.9	< 0.2[a]	0.7 ± 0.5 (43)
o,p'- DDT	13.6 ± 6.6	< 0.2[a]	< 0.2[a]
p,p' -DDD	43.1 ± 16.2	< 0.2[a]	1.1 ± 0.6 (45)
o,p' -DDD	17.0 ± 0.0 (20)	< 0.2[a]	< 0.2[a]
SumDDT	844.3 ± 471.3	1.4 ± 0.9	8.0 ± 9.6*
DDTs/ PCBs	1.9 ± 0.5†	0.1 ± 0.1	0.4 ± 0.4

*significantly greater concentration in pup blood than in dam blood, $p < 0.001$

†significantly greater than in dam blood

[a]all samples below this detection limit

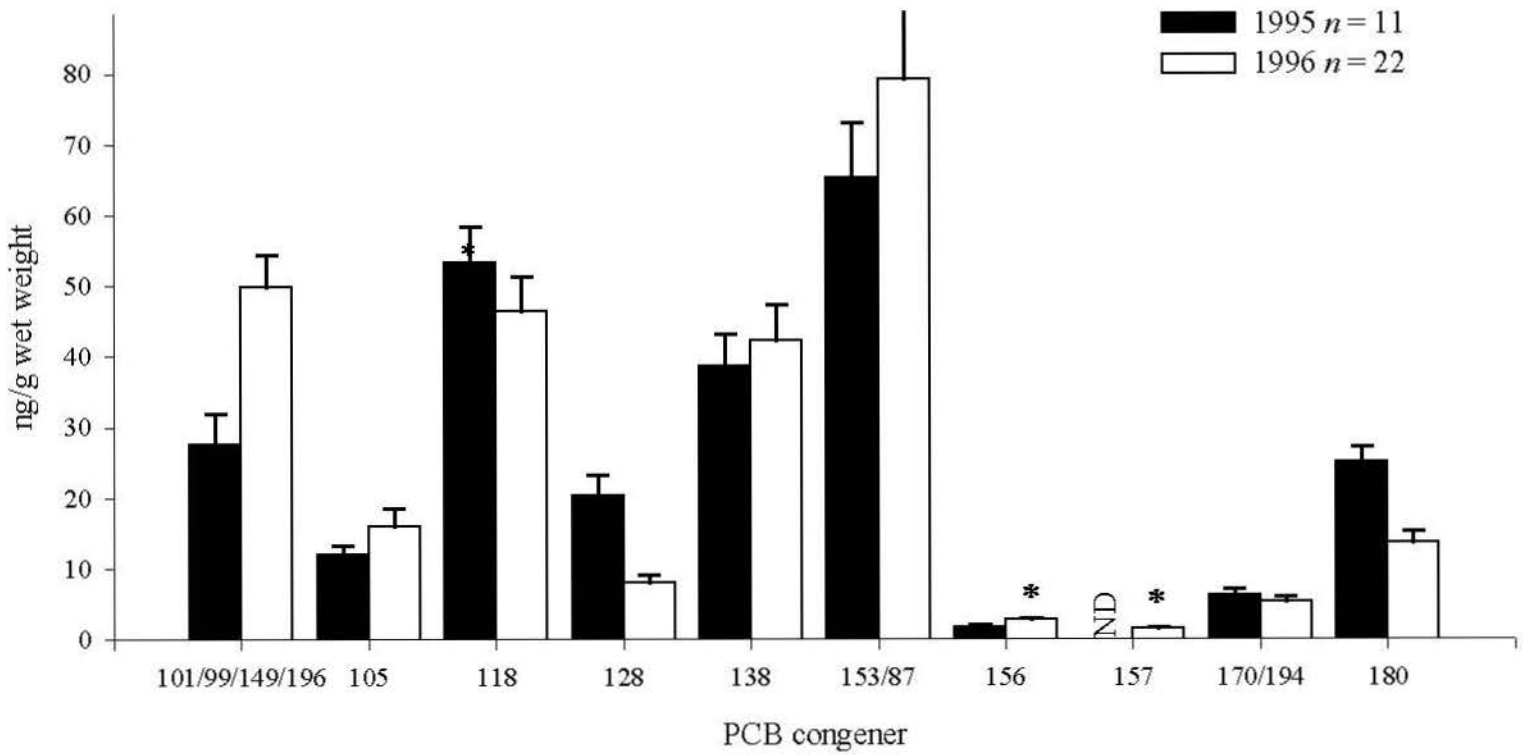

Fig 2.1. Mean ± SE PCB congener concentrations (ng/g wet weight) in early lactation northern fur seal milk collected in 1995 and 1996 on St. George Island, Alaska.
* significantly greater concentration, $p < 0.05$. ND not detected in 1995 samples

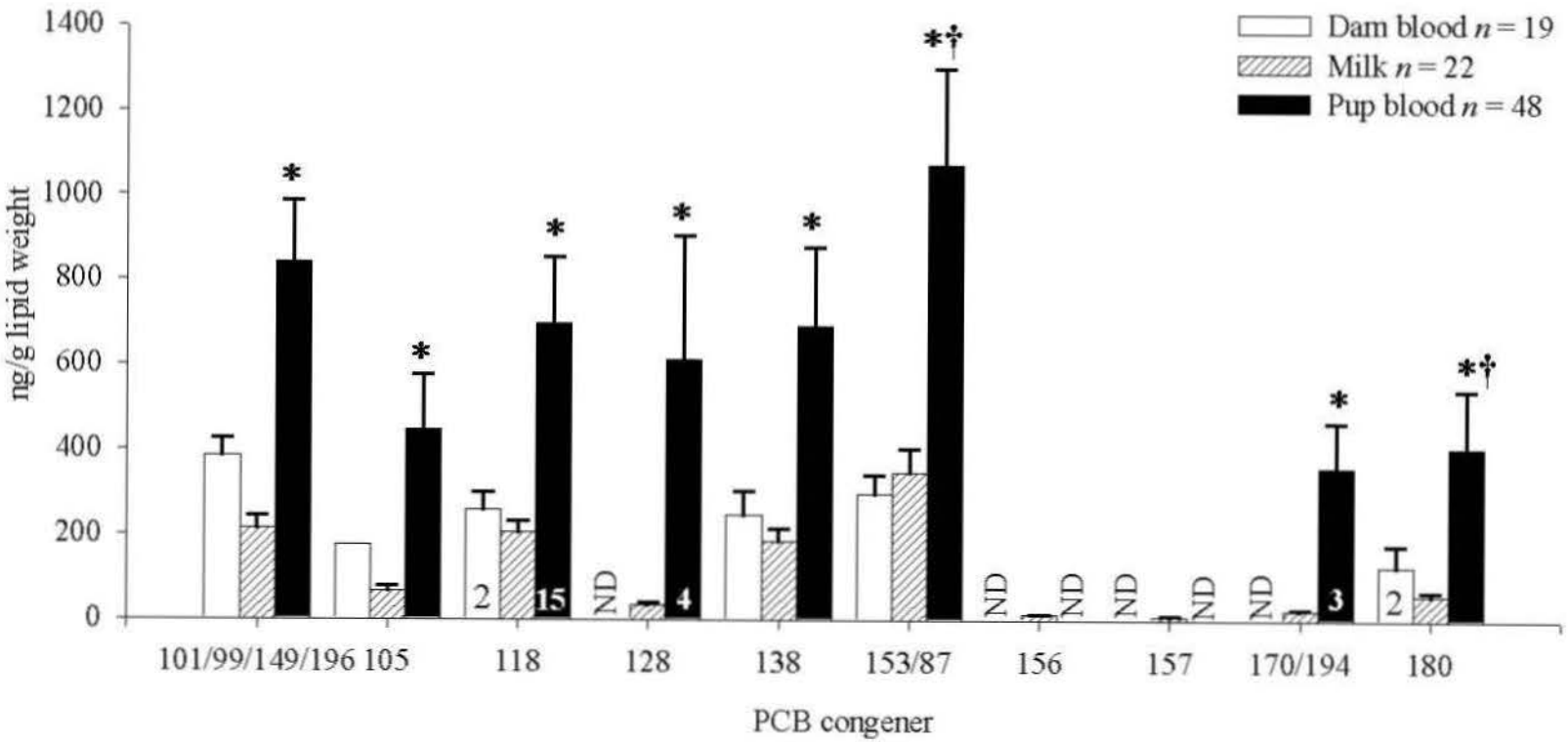

Fig 2.2. Mean ± SE PCB congener concentrations (ng/g lipid weight) in blood and early lactation milk of northern fur seal dams and pups on St. George Island, Alaska, 1996.

Digits within bar indicate number of samples above detection level when > 50% of samples were below detection level

*indicates significantly greater concentration than in milk, $p < 0.01$

†significantly greater concentration than in dam blood, $p < 0.003$

ND indicates not detected in this blood group

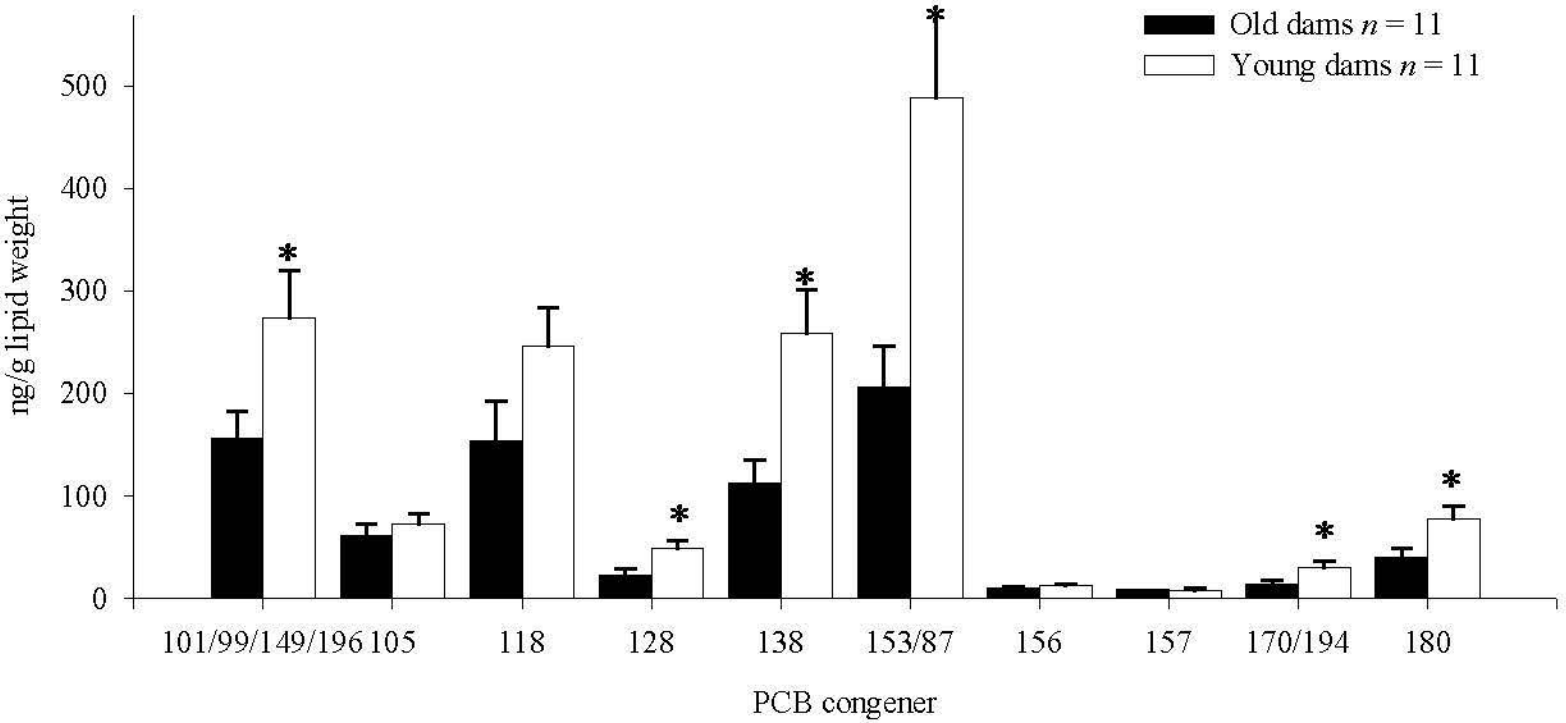

Fig 2.3. Mean ± SE PCB congener concentrations (ng/g lipid weight) detected in early lactation milk of northern fur seals grouped by relative age from St. George Island, Alaska, 1996.
*indicates significantly greater concentration at $p < 0.05$

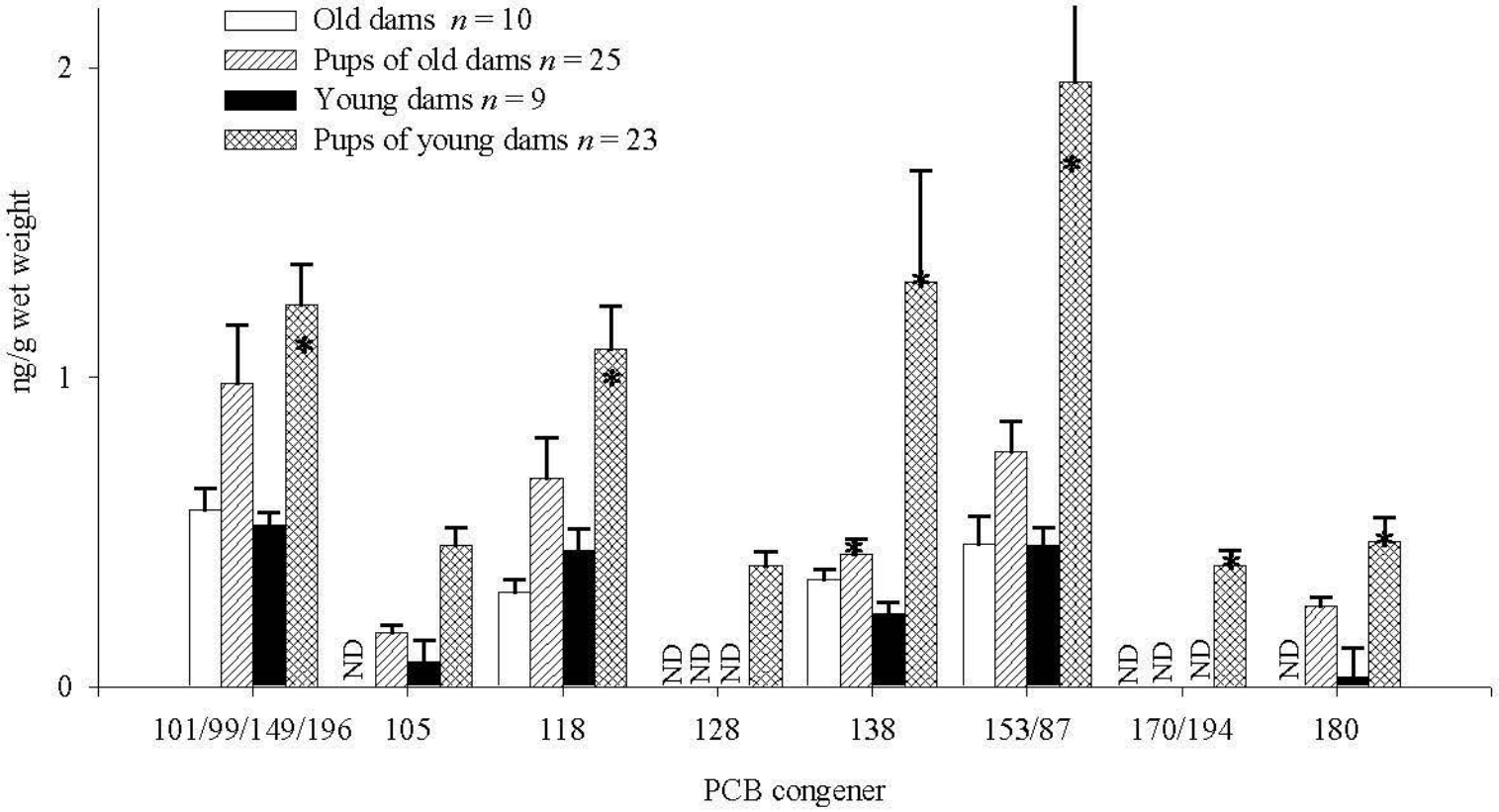

Fig 2.4. Mean ± SE PCB congener concentrations (ng/g wet weight) in northern fur seal blood from 19 early lactation dams and 48 pups grouped by relative dam age on St. George Island, Alaska in 1996. *indicates significantly greater conentration than pups of old dams and all dams regardless of age, $p < 0.001$ ND indicates not detected in group

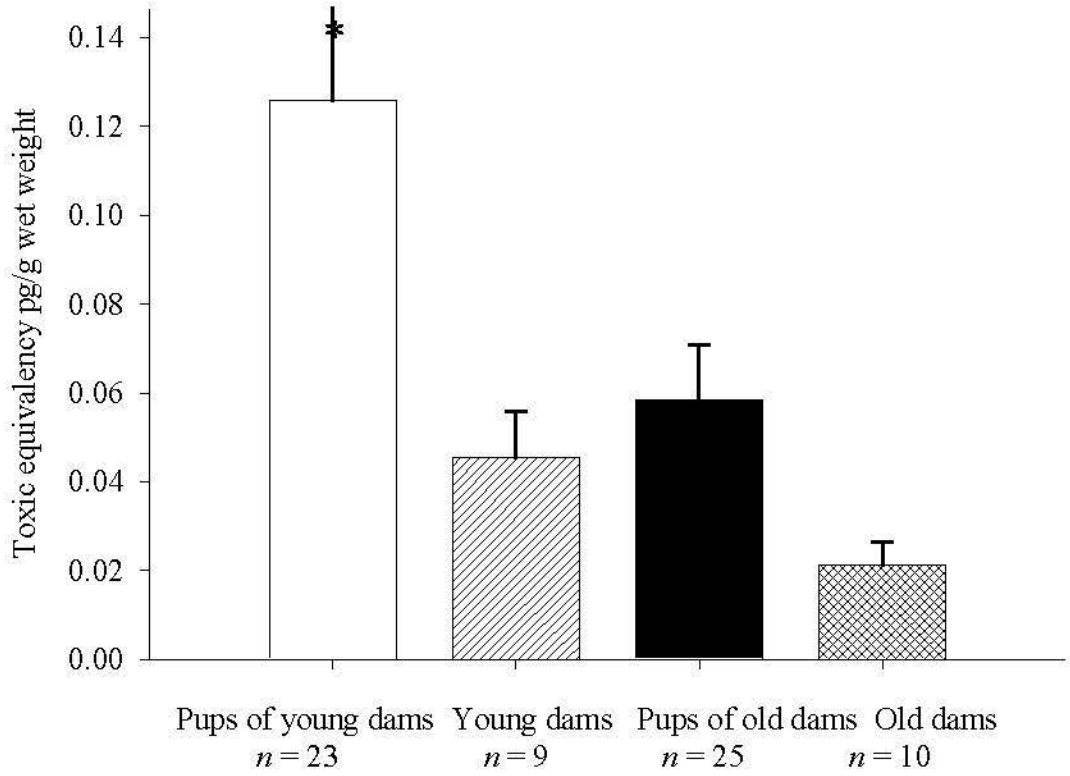

Fig 2.5. Mean ± SE TCDD TEQ (pg/g wet weight) in northern fur seal dam and pup blood grouped by age of dam from St. George Island, Alaska in 1996.
*signficantly greater than pups of old dams and all dams regardless of age at $p = 0.002$

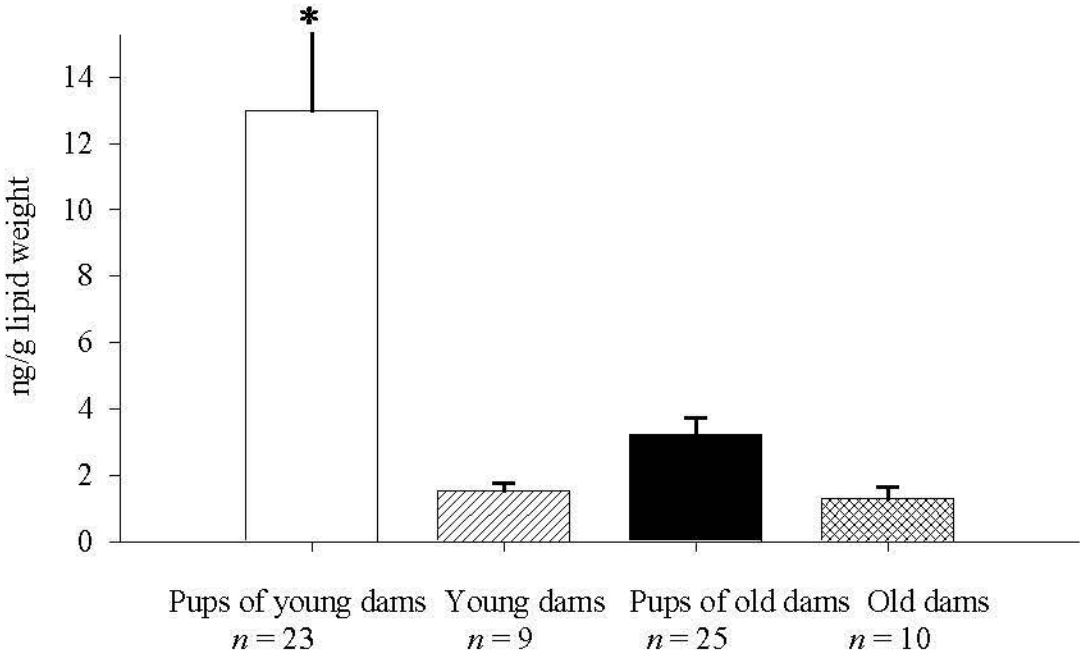

Fig 2.6. Mean ± SE concentrations of *p,p'*-DDE (ng/g wet weight) in northern fur seal dam and pup blood grouped by age of dam from St. George Island, Alaska, 1996. *indicates signficantly greater than pups of old dam and all dam regardless of age, $p < 0.02$

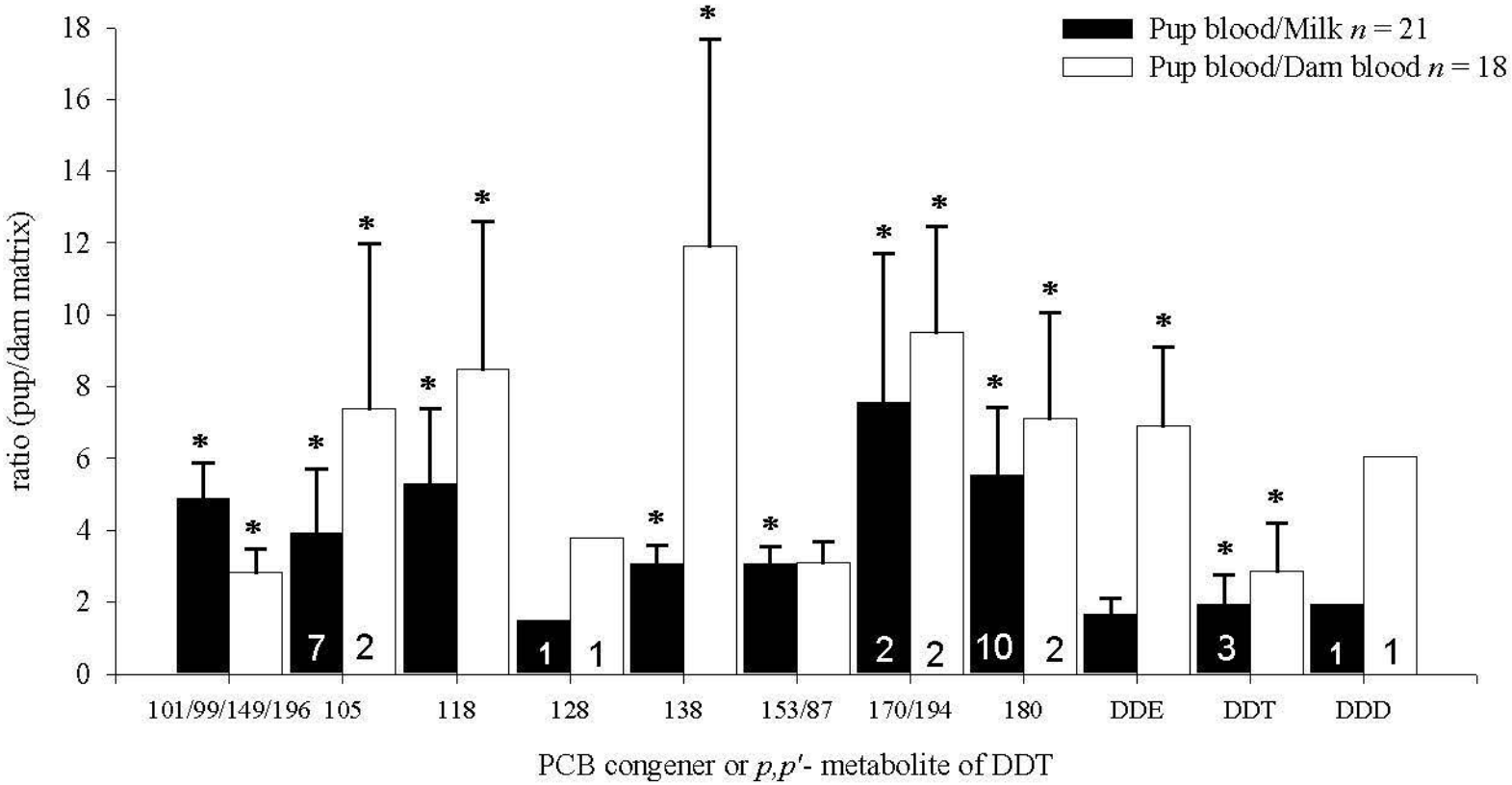

Fig 2.7. Mean ± SE "biomagnification factor" for pup blood from milk or dam blood for PCB congeners or *p,p'*- metabolites of DDT (l.w.) for northern fur seal pup and dam pairs on St. George Island, Alaska in 1996. *indicates minimum value of the 95% confidence interval is >1.0. Digits within bars are the number of samples with measurable levels when > 50% of blood samples were below detection level.

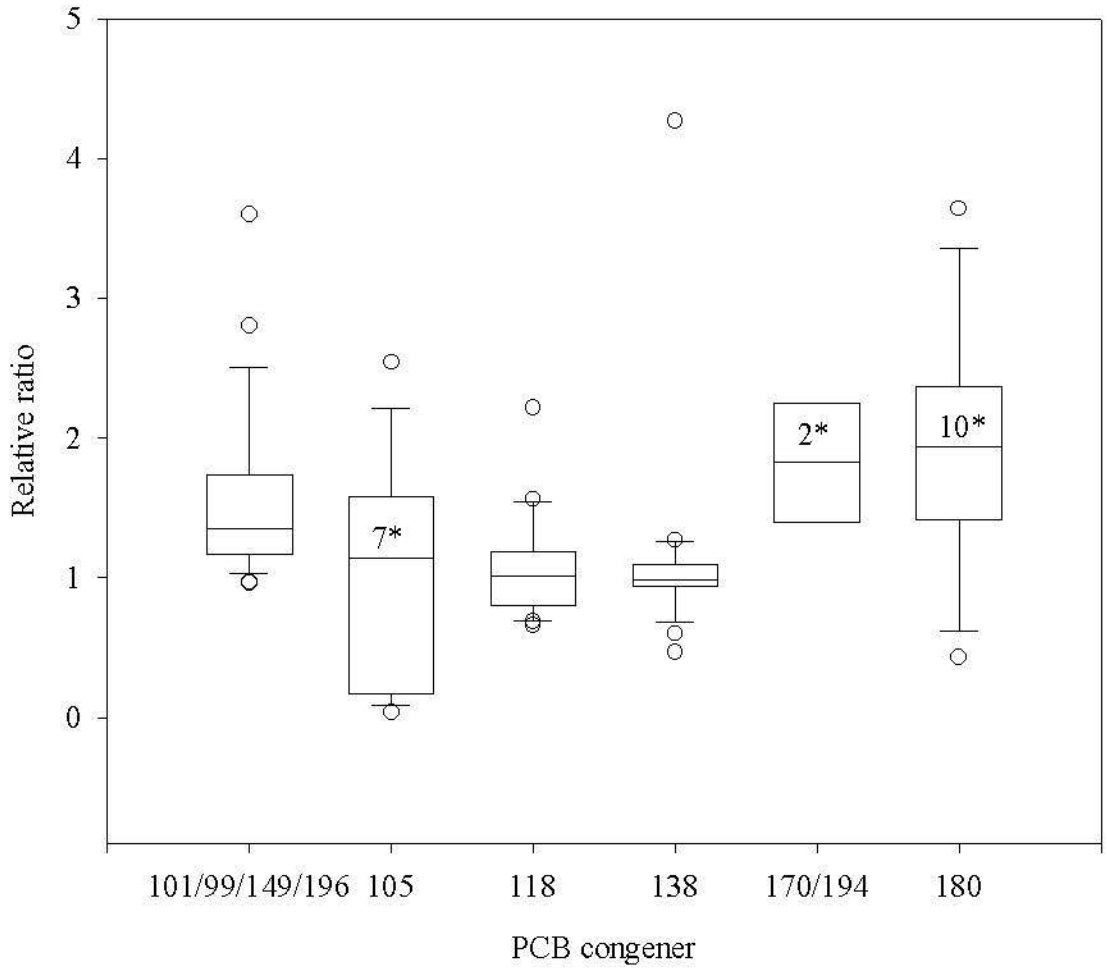

Fig 2.8. Relative ratio expression of bioaccumulation from northern fur seal milk to pup blood on PCB congener concentrations normalized to the concentration of CB-153 in 21 dam/pup pairs using the formula:

$$Rrel(CBx)=R_{153}(CBx)\text{pup blood}/R_{153}(CBx)\text{milk}$$

*Digits within boxes are the number of samples with measurable levels when > 50% of blood samples were below detection level. 95% confidence interval > 1.0. Boundaries of boxes are the 25th and 75th percentiles, the line is the median and the whiskers are the 5th and 95th percentiles, outliers are displayed as open circles.

Chapter 3

Age-dependent Effects of Organochlorine Contaminant Exposure on Hematological and Immune Functional Assays in Free-Ranging Northern Fur Seal Pups (*Callorhinus ursinus*)

3.0 Introduction

Breeding rookeries for more than 72% of the world's population of northern fur seals (*Callorhinus ursinus*) are located in the Bering Sea on the two largest Pribilof Islands, St. Paul and St. George, Alaska (Loughlin et al., 1994). The current Pribilof stock abundance is less than half of historical levels and is listed as depleted under the Marine Mammal Protection Act (Loughlin et al., 1994; York et al., 1997). Until 1996, the St. George subpopulation underwent an unexplained decline of 4-6% per year for more than a decade (York et al., 1997). Long-term monitoring of population trends suggest that the decline was due, at least in part, to increased post-weaning mortality at sea (Trites and Larkin, 1989; Trites, 1992). The cause(s) of the increased mortality is unknown.

Organochlorine (OC) contaminant exposure has been documented in the Pribilof population of northern fur seals through tissue residue analyses, but the potential effects of OC exposure on health or survival has not been investigated (Anas and Wilson, 1970a; Anas and Wilson, 1970b; Kurtz and Kim, 1976; Calambokidis and Peard, 1985; Calambokidis, 1987; Bacon et al., 1992; Varansi et al., 1992; National Marine Fisheries Service, 1993; Schantz et al., 1993; Mössner et al., 1994; Becker et al., 1997; Krahn et al., 1997).

Organochlorine compounds including polychlorinated biphenyls (PCBs), 1,1-bis[*p*-chlorophenyl]-2,2,2-trichloroethane (DDT) and their metabolites are ubiquitous environmental contaminants that elicit a broad spectrum of biological effects in animals (Tanabe et al., 1994; Safe, 1994). High doses of these compounds are acutely toxic and chronic exposure to low levels currently found in the environment may induce adverse effects including immunosuppression in mammals and birds (Vos, 1977; Safe, 1984). Low-level exposure to these contaminants may cause death indirectly through increasing susceptibility to opportunistic pathogens. In vertebrates, the type and degree of effect on the immune system is time, species and dose-dependent (for reviews see Vos and Luster, 1989 and Vos et al., 1996). Immunotoxic effects of PCBs on both cell-mediated and humoral immune function are described in humans and in a variety of laboratory species including guinea pigs, rats, mice, rabbits, chickens and monkeys (Chang et al., 1981; 1982; Thomas and Hinsdill, 1978; 1980; Thomas and Faith, 1985 and Vos et al., 1996). Exposure to DDT and its metabolites also cause immunosuppression in rats and increased mortality with exposure to duck hepatitis virus in ducks (Friend and Trainer, 1972; Banerjee et al., 1996).

The developing mammalian immune system is particularly susceptible to damage from low level chemical exposure during the perinatal period (Carstens et al., 1979; Thomas and Hinsdill, 1980; Bleavins et al., 1984; Safe, 1984; Thomas and Faith, 1985; Guo et al., 1995; Pluim et al., 1996; Hansen, 1998). During the perinatal period the immune defense mechanisms are underdeveloped and not readily activated thus increasing vulnerability to infection even without chemical exposure (Nehlesen-

Cannarella and Chang, 1992; Xanthou, 1993; Ellis et al., 1997). Prenatal and neonatal animals can manifest immunotoxicity at PCB exposure levels with no detectable affect on adults (Linder et al., 1974; Carstens et al., 1979; Gallenberg and Vodicnik, 1989; Ross et al., 1997). Pre- and post-natal human exposure to PCBs at low levels, considered to be "background", is associated with decreased monocyte and granulocyte counts at three months of age suggestive of PCB influences on development of the human immune system (Weisglas-Kuperus et al., 1995). Prenatal exposure to rats born and raised by dams fed a continuous diet of Baltic fish oil, naturally contaminated with complex PCB mixtures, have impaired immune responses (Ross et al., 1997). Non-specific immune function effects in the rat pups were characterized by impaired mitogen-induced T-lymphocyte proliferative responses and thymus-related effects, suggesting that developing thymocytes were targeted as well as specific antibody response impairment (Ross et al., 1997). When female rhesus monkeys were orally exposed to 5 to 40 µg/kg body weight of Aroclor 1254 daily before breeding, during pregnancy and lactation, their offspring had reduced IgM production in response to sensitization to sheep red blood cells at 22 weeks of age compared to unexposed controls (Arnold et al., 1995). When tested at 28 and 60 weeks of age, a decrease *in vitro* lymphocyte proliferation response to mitogen (ConA) was detected. There were no statistical differences for exposed versus unexposed monkeys using other mitogens (PHA or PWM) or in the NK cell assays and mixed lymphocyte cultures. In contrast, maternal exposure to a single IP dose of 300 mg/kg Aroclor 1254 prior to breeding in white-footed mice (*Peromyscus leucopus*), increased the lymphoproliferative responses to PHA in early weaned (3 weeks) pups

examined at 6 weeks of age (Wu et al., 1999). The white-footed mouse pups also had significant decreases in body weight, ratio of spleen weight to body weight, numbers of peripheral white blood cells and lymphocytes, and numbers of monocytes than unexposed pups. White-footed mouse pups weaned and examined at 4 weeks also had low body weights, liver weight, lymphocytes and had lower serum antibody responses to 2,4-dinitrophenol keyhole limpet hemocyanin antigen injections than unexposed controls (Wu et al., 1999).

Many toxic effects, including immunotoxicity, of the non-*ortho* and mono-*ortho* substituted or 'dioxin-like' PCBs are thought to be initiated through binding to the aryl hydrocarbon (*Ah*) or TCDD (tetrachlorodibenzo-*para*-dioxin) receptor (Safe, 1994). Some PCB congeners are assigned a relative potency to TCDD (assigned the value of one) called a 'toxic equivalency factor' or TEF. These are multiplied by the concentrations of the individual congeners in a sample and summed to calculate the 'dioxin-like' toxic equivalency quotient or TEQ (Safe, 1990). The TEQs can then be used comparatively in a risk assessment of exposure. Unfortunately there are extremely large interspecies differences in the sensitivity to the toxicity of PCBs and DDTs which has complicated risk assessment in humans and free-ranging wildlife (Vos et al., 1996). Higher chlorinated PCB mixtures are more potent immunosuppressants than the lower chlorinated mixtures in laboratory animals (Tryphonas, 1994) yet the highest TEFs are for lower chlorinated coplanar congeners (Safe, 1990). Not all PCB congener immunotoxic effects are *Ah*-receptor mediated. Some OC-induced effects are partly or entirely non-*Ah*-receptor dependent, including neurological effects, developmental effects, and vitamin A

and thyroid deficiencies (Brouwer et al., 1986b; Morse et al., 1992; Lans et al., 1993; Hooper and Clark, 1995). Presently, TEF calculation schemes erroneously assume additive effects of congeners in the mixture. In fact, certain PCBs may antagonize the immunotoxic effects of other chemicals including dioxin (Davis and Safe, 1989). Low level exposure to PCB and polychlorinated dibenzo-*p*-dioxins/furans *in vitro* augmented rat lymphoproliferative and splenocyte responses to T-cell mitogens (Omara et al., 1997). Thus, the interactions are quite complex confounding the interpretation of field studies.

Free-ranging marine mammals are vulnerable to long term PCB toxicosis (Tanabe, 1988). As a result of their high trophic level, some species of marine mammals accumulate high levels of OC compounds, especially in their lipid rich tissues like blubber (Bowes and Jonkel, 1975; Reijnders, 1980; Tanabe et al., 1981; Tanabe et al., 1984; Tanabe and Tatsukawa, 1991; Norstrom and Muir, 1994; Tanabe et al., 1994). Even with extraordinary high tissue burdens of contaminants, it is difficult to conclusively demonstrate contaminant-induced lesions in free-ranging marine mammals.

Contaminant-induced immunosuppression has been speculated to be a contributing factor to the extraordinarily high mortality experienced by several marine mammal species during recent morbillivirus epizootics (Hall et al., 1992; Simmonds et al., 1993; Aguilar and Borrell, 1994a). A series of publications from a controlled study in captive juvenile harbor seals (*Phoca vitulina*) fed wild-caught OC contaminated fish have documented a decrease in cellular and humoral immune responses in connection with dietary contaminants exposure and measured blubber OC levels (De Swart et al., 1994; Ross et al., 1995; De Swart et al., 1995a; Ross et al., 1996). A report on five free-

ranging bottlenose dolphins (*Tursiops truncatus*) indicated a negative correlation between blood PCB levels and T-lymphocyte responses to mitogen stimulation (Lahvis et al., 1995). From the evidence presently available, it appears that persistent environmental contaminants may represent a risk to the health of free-ranging marine mammals both in North America and in Europe (Ross et al., 1996).

An unexplained decline in the St. George Island population of northern fur seals prompted this study to evaluate the potential effects of organochlorine (OC) contaminant exposure on immune function in a cohort of free-ranging pups. I tested the hypothesis that pups with higher exposure to OCs and levels in whole blood would demonstrate hematological or immunological differences from pups with lower exposure either as neonates or one to two months later in the nursing period.

3.1 Materials and Methods

Study site

Northern fur seals were captured on four of the six rookery beaches of St. George Island, Alaska (N56° 34’ W169° 41’), one of the two main rookery islands in the Pribilof Island group in the Bering Sea. The treeless, wind-swept sub-arctic island is located 31 km from the continental shelf break. The rookery beaches are composed of sand, gravel and boulders of volcanic origin. During the fur seal occupation from May to November, the weather is typically overcast and marked by rain, fog with mean air and water temperatures around 5°C.

Animal criteria, capture, and marking techniques

Northern fur seal neonates were captured between 17 July – 5 August 1995 (*n* = 20) and 13 July – 3 August 1996 (*n* = 50), with a noose pole extended from a 3-person roving 'blind box' constructed of plywood (approximately 1x1.5x2m) using methods detailed elsewhere (Antonelis, 1992; Boltnev et al., 1998). This 'blind box' afforded protection of personnel from aggressive territorial bulls and allowed access to most of the rookery with minimal animal disturbance.

All neonates were accompanied by their dams at the initial capture and dams were classified as young ($\leq$ 5 years of age and likely to be primiparous) or old (> 7 years, assumed to be multiparous) by body size, pelage and vibrissae characteristics (Vladimirov and Nikulin, 1993). The vast majority of neonates included in the study were estimated to be less than seven days old, based on the condition of the umbilical cord remnant or degree of umbilical healing, neonatal behavior and observation of characteristic periparturient/pre-estrus behavior of the dam (Gentry, 1998). Although neonates were observed in contact with their dams when selected for capture, the timing, duration and volume of their most recent suckling bout was not known.

Prior to release in 1996, each neonate was given 1 ml of tetanus toxoid (Tetanus Toxoid with MetaStim™ adjuvant, #277302A, Fort Dodge Laboratories, Fort Dodge IA 50501) in the right shoulder musculature. To facilitate identification of individuals, each neonate in 1996 was marked with a unique symbol by clipping the guard hairs on the top of the head and applying gel hair bleach (Clairol Beyond Blond®). Blood was collected in both years as described below.

A cohort of 44 pups of the 1996 group was recaptured at intervals ranging from 29 to 51 days later (mean 39 ± 6) for repeat blood collection (between 24 August and 12 September 1996). Pups were recaptured either individually by stealth using a net or by herding all pups on a section of the rookery.

Blood sample collection and measurements

Seals were physically restrained for blood collection in a neoprene vest secured with Velcro® tabs, resting in ventral recumbancy on a V-shaped plywood platform. Blood samples were obtained from a superficial plantar flipper vein (2 samples were drawn from the caudal gluteal vein via syringe) using a 21 gauge Venoset® blood collection set into at least three types of evacuated tubes: Venoject II® 5 ml plastic tubes containing dry ethylenediaminetetraacetic acid (EDTA) as an anticoagulant (for complete blood cell counts and OC determinations), Terumo® serum separator tubes (both by Terumo Medical Corp. Elkton MD) for the harvest of serum and 8 ml draw Vacutainer® CPT™ cell preparation tubes with sodium citrate used to isolated lymphocytes for immune function assays (Becton-Dickinson and Company, Franklin Lakes NJ 07417). After gentle mixing, 5 ml of EDTA whole blood was transferred to a methylene chloride-rinsed glass vial with a Teflon lined lid for OC determination and frozen (to less than -20°C) within 6 hours of collection. Serum was obtained after clotting and centrifugation of a Terumo® serum separator tube. The harvested serum was transferred to cryogenic vials and frozen to less than -70°C within 6 hours of collection until thawed for the serological assays described below.

Capture and sampling activities were conducted under Marine Mammal Protection Act/Fur Seal Act permits #837 or #1003 and a research protocol approved by the Institutional Animal Care and Use Committee of the University of Alaska Fairbanks.

Hematology

Hematological values were determined from EDTA anticoagulated blood as follows: hematocrit was determined by measuring packed red cells as a percent of blood volume after centrifugation in a microhematocrit tube at 10,000 x g for 3 minutes. Total protein was determined from the resultant supernatant plasma with a hand-held refractometer (AO Scientific Instruments, Buffalo NY 14215). The total leukocyte count was determined manually using the Unopette® system (Becton-Dickinson and Company, Franklin Lakes NJ 07417) and a hemocytometer (Bright-Line, AO Scientific Instruments, Buffalo NY 14215). The differential cell counts were made manually under oil immersion microscopy on fresh blood smears stained with Wright's-Giemsa type stain (Dip Quick Stain, Jorgensen Laboratories, Loveland CO 80538) by identifying 100 leukocytes based on morphology.

Cell-mediated immune function via mitogen-induced lymphocyte proliferation assay

Peripheral blood mononuclear leukocytes (PBML) were obtained from whole blood in the Vacutainer® CPT™ cell preparation tubes. PBML were harvested by the following technique and cryopreserved by a method adapted from Truax et al. (1990). Cell preparation tubes were centrifuged in a swinging bucket rotor for 20 minutes at 1800 x g within 8 hours of blood collection. The supernatant plasma plus anticoagulant fluid were aspirated and discarded to within 4 mm of the layer consisting of PBML, a 2-4 mm

thick layer which could be visualized just a few mm above the separator gel. The PBML layer was gently suspended with 5 ml of a sterile chilled phosphate buffered saline (PBS, adjusted to pH 7.2), transferred into a 15 ml sterile plastic screw cap centrifuge tube (Corning Incorporated, Corning NY 14831) and brought to a volume of 15 ml by addition of PBS. Tubes were gently inverted twice to wash and distribute cells and were centrifuged again at 250 x g for 10 minutes. The supernatant buffer was aspirated to be discarded and the cell pellet was re-suspended in 1 ml of a cryopreservation media consisting of 40 parts fetal bovine serum (FBS, Sigma Chemical, St. Louis MO 63178), 5 parts DMEM (Dulbecco's Modified Eagle Medium without Ca^{++} or Mg^{++}, Gibco-BRL, Grand Island NY 14072) and 5 parts dimethylsulfoxide (DMSO). This suspension was then transferred into sterile cryogenic vials (Nalgene Company, Rochester NY 14602) and kept cool. Cryogenic vials were placed in an absolute isopropyl alcohol bath controlled-rate freezing container (Cryocooler, Nalgene Company) and then packed in pulverized dry ice or placed in a –70°C freezer to be cooled at 1°C per minute for a minimum of four hours. Vials were temporarily held at –70°C prior to transfer into liquid nitrogen where they were subsequently held until analysis.

The lymphocyte proliferation assays were conducted later at the Marine Mammal Immunology Laboratory at the University of California Davis, Davis CA. Briefly, PBML were thawed rapidly in a 37°C water bath and re-suspended in 13 ml PBS. Cells were isolated by centrifugation at 200 x g for 8 minutes and re-suspended in media solution consisting of DMEM, 1% 100 mM sodium pyruvate, 2% 200mM L-glutamine, 1% 10 mM non-essential amino acids, 1% 500IU penicillin/5000IU streptomycin (all from

Gibco-BRL) and 10 % fetal bovine serum. A live/dead cell ratio and cell numbers were calculated for each sample using a 1:10 dilution in a vital stain (ethidium bromide/acridine orange solution) and a hemocytometer. Samples were diluted in the media to achieve $2x10^6$ cells/ml and 100 µl were aliquoted to appropriate wells of a 96-well plate to achieve $2x10^5$ cells per well. Three mitogens were used to stimulate cell proliferation: concanavalin A (ConA), pokeweed (PWM) and phytohemagglutinin (PHA) (Sigma Chemical), diluted in media to a concentration of 2.5 and 0.5 µg/ml. A volume of 100 µl of appropriate mitogen or media control was added to the appropriate wells. Plates were incubated at 37^oC for 72 hours. When a sample vial did not yield sufficient live cell numbers to allow testing with all three mitogens at both dilutions ConA was given preference. For each individual seal, both perinatal capture and recapture samples were run at the same time on the same plate to eliminate run to run variability. A colorimetric BrdU (5-bromo-2'-deoxyuridine) enzyme-linked immunosorbant assay (ELISA) was employed according to manufacturer instructions (#1 647 229, Boehringer Mannheim, Indianapolis IN 46251) to quantify the cell proliferation based on the measurement of BrdU incorporation during DNA synthesis after an 18 hour, 37^oC incubation period. An automated ELISA plate reader (UVmax, Kinetic Microplate Reader, Molecular Devices) was used to determine the optical density at 450 nm of the individual sample wells after washing, addition of conjugate and substrate solutions per kit instructions. The optical density divided by the media control was used to determine the stimulation index and reported as SI. An SI of 0 to 2.0 was considered a poor proliferative response, 2.1 to 4.0

was a moderate response and 4.1 or greater was a good response based on previous experience with the kit for a variety of marine mammal species.

Haptoglobin serum levels

Serum haptoglobin levels were determined using a commercial gel electrophoresis kit and haptoglobin-hemoglobin binding assay (Titan Gel High Resolution Protein Kit #3040, Helena Laboratories, Beaumont TX 77704). A 15% solution of northern fur seal hemoglobin was obtained by dilution of washed red blood cells that had been lysed in sterile water and by freezing. Two µl of the 15% hemoglobin solution was added to each 38 µl of test sample serum in a microcentrifuge vial and mixed by vortex. Two µl of the mixture was placed on the gel previously prepared according to kit instructions. Electrophoresis was conducted for 45 minutes at 105 volts. Gels were stained with an o-dianisidine solution for ten minutes and rinsed three times according to the kit specifications. Haptoglobin concentrations were calculated from the optical density of the migrating haptoglobin bound to hemoglobin band minus the density of the free hemoglobin band from gels after air drying overnight using a Hewlett Packard transilluminating scanner and software package, SigmaGel (SPSS, Chicago IL). Results are expressed in mg of hemoglobin binding capacity per 100 ml of serum.

Humoral immunity-tetanus antibody response

Tetanus antibody responses were determined from pre and post-injection serum by an ELISA using the methods described by Ham-Lamme' et al. (1999). Briefly, serum samples were diluted to four concentrations from 1:10 to 1:80 as well as a 6-2 fold dilution of a fur seal standard obtained from a sensitized captive adult female and an

equine standard (diluted 4-2 fold). The stock tetanus toxoid antigen (East Coast Biologics Inc.) was diluted to 5 µg/ml in a bicarbonate coating buffer then applied to wells in a 96-well plate and incubated overnight in a refrigerator. Excess antigen was washed with a phosphate buffered saline solution containing 1% Tween 20. A blocking step was applied for two hours with 3% bovine serum albumin (BSA, Sigma Chemical) in wash buffer at 200 µl per well. Wells were washed twice and 100 µl of diluted test serum added to each appropriate well and incubated at room temperature for one hour then washed again. A volume of 100 µl of Protein A-HRP (1:1500 in wash buffer, Bio-Rad, Hercules, CA) was added to each well and the incubation continued for one hour. Substrate was prepared from one o-phenylenediamine tablet (Sigma Chemical) dissolved in 12 ml of citrate buffer and adding 5 µl of H_2O_2. After a wash, 100 µl of substrate was added to each well and incubation continued for 20 minutes. Finally, 150 µl of a stop reagent (1 M H_2SO_4) was added and the optical density determined at a wavelength of 490 nm (UV Max, Kinetic Microplate Reader, Molecular Devices). This assay was conducted at the Marine Mammal Immunology Laboratory at the University of California Davis, Davis CA.

Total immunoglobulin levels

Total immunoglobulin levels (Ig) were determined on serum samples by standard radioimmunodiffusion methods. First, fur seal immunoglobulin was purified with a two step procedure employing caprylic acid and ammonium sulfate as previously described by McKinney and Parkenson (1987). Rabbits were given a two immunizations of the purified immunoglobulin; the first with Freund's Complete Adjuvant and second with

Freund's Incomplete Adjuvant (Gibco/BRL, Grand Island, NY) before serum was collected. Reactivity of the polyclonal antiserum was evaluated by immunoelectrophoresis. Fur seal immunoglobulin preparations were further purified with Protein A and immunoglobulin concentration was determined using a BCA Protein Assay (Pierce, Rockford, IL) per manufacturer's instructions. Bovine Ig (stock of 2mg/ml; Pierce) was used for the standard curve. The Protein A purified fur seal Ig preparation was then diluted appropriately in phosphate buffered saline containing 7% bovine serum albumin (PBS/BSA - Sigma, St. Louis, Mo) to obtain concentrations of 750, 500, 250 and 125 µg/ml. Single radial immunodiffusions were performed as previously described Lu and Miller (1996). Briefly, agarose plates were prepared using a 1.2% Nobel Agar (Difco Labs, Detroit, MI) and 3% PEG (6,000 wt, The Binding Site, Birmingham, UK) in a Tris/Boric Acid Buffer (0.09M Tris, 0.09M Boric Acid, 0.003M EDTA, pH 8.3). Polyclonal antibody was titrated to determine optimum gel concentration and added appropriately. Unknown fur seal serum samples were diluted in PBS/BSA. Five microliters of above described purified Ig preparations, diluted unknown and diluted, pooled serum sample (serving as a positive control) was added to wells; unknowns were plated in duplicate. The diameters of the resulting precipitant rings were measured and the diameter values were squared. Diameter values for purified fur seal immunoglobulin were plotted to establish a standard curve (Lu and Miller, 1996). Unknown and serum control values were determined based on the derived standard curve and results are reported in mg/ml. This assay was also conducted at the Marine Mammal Immunology Laboratory.

Analytical techniques for organochlorine contaminants

Concentrations in whole blood of selected PCB congeners and other OC compounds were determined by the Environmental Conservation Division of the Northwest Fisheries Science Center (NWFSC), National Marine Fisheries Service, Seattle WA, using rapid high-performance liquid chromatography (HPLC) coupled with a photodiode array detection method as previously described (Krahn et al., 1994). Briefly, 3-4 g of whole blood was thawed and weighed to the nearest 0.01 g then homogenized with 20 ml of hexane/pentane (1:1 v/v), sodium sulfate and the surrogate standard (1,7,8-trichlorodibenzo-*para*-dioxin; 250 ng), centrifuged and decanted into a concentrator tube. The homogenization process was repeated on the sample remaining in the centrifuge tube, and the extracts were combined. A 1 ml portion of extract was removed for lipid quantitation described below. The remaining sample extract was evaporated down to a volume of approximately 1 ml. The sample extract was loaded onto a custom gravity-flow cleanup column (which contained a glass wool plug, silica gel, basic silica gel and acidic silica gel) to separate the CBs from other interfering compounds (i.e. lipids, aromatic hydrocarbons). The CBs were eluted from the cleanup column with 14 ml of hexane/methylene chloride (1:1 v/v) and collected into a concentrator tube. The HPLC internal standard was added to each sample (1,2,3,4-tetrachlorodibenzo-*para*-dioxin; 250 ng) and the solvent volume was reduced to approximately 150 µl under nitrogen evaporation. Eleven dioxin-like congeners (CBs -77, -81, -105, -118, -126, -156, -157, -169, -170, -180, and -189) were resolved from other selected CBs (CBs -101, -128, -138, and -153), hexachlorobenzene (HCB) and chlorinated hydrocarbons (e.g. *p,p'*-DDD, *p,p'*-

DDE, *p,p'*-DDT) by HPLC on 2 (1-pyrenyl) ethyldimethylsilylated silica (PYE) analytical columns (connected in series) cooled to 9°C and were detected with a Waters 996 PDA detector (Waters, Milford, MA). These analytes were identified by comparing their UV spectra (200-310 nm) and retention times to reference standards (Krahn et al., 1994). Compound purity was confirmed by comparing UV spectra collected for a peak to the apex spectrum. In this method there is co-elution of the minor CBs -99/149/196 with possibly others and CB-101; and CB-87 with CB-153; and CB-194 with CB-170 (Krahn et al., 1994).

Lipid determination

Total lipid concentrations of whole blood samples were determined by thin layer chromatography with flame ionization detection (TLC/FID) using an Iatroscan Mark 5 (Iatron Laboratories, Tokyo, Japan) at the NWFSC laboratory. The extracts were spotted on Chromarods (Type SIII) and developed in a solvent system containing 60:10:0.02 hexane:diethyl ether:formic acid (v/v/v). Various classes of lipids (i.e., wax esters, triglycerides, free fatty acids, cholesterol, and phospholipids) were separated based on polarity, with the nonpolar compounds (i.e., wax esters) eluting first followed by the more polar lipids (i.e., phospholipids). The Iatroscan was operated with a hydrogen flow rate of 160 ml/min and air flow of 2000 ml/min. Data was acquired and analyzed using TDatascan software (RSS Inc., Bemis, TN). A four-point linear external calibration was used for quantitation. Total lipid concentrations were calculated by adding the concentrations of the five lipid classes for each sample and were reported as percent total lipid. Duplicate TLC/FID analyses were performed for each sample extract and the mean

value reported. The lipid-adjusted congener concentrations were calculated on the basis of the percent lipid content of the individual blood sample: (concentration of CB_x in ng/g w.w.) / (percent lipid of sample / 100) and the results were reported as lipid weight (l.w.).

Calculated values

Using the areas under the congener curves and concentrations determined from the HPLC/PDA chromatograms, the total CB concentrations were calculated by the following formula:

Total CBs = sum of concentrations of selected CB + sum concentrations of other CBs. Individual analyte and total CB concentrations were reported as ng/g wet weight. The 'dioxin-like' toxic equivalency quotients (TCDD TEQs) were calculated using an additive model of toxicity (Safe, 1990). In this method, molar concentrations of the dioxin-like congeners were multiplied by their toxic equivalency factors (TEFs) recently recommended by the World Health Organization-European Centre for Environment and Health (WHO-ECEH) and the International Programme on Chemical Safety (IPCS) (Ahlborg et al., 1994). When the concentration of a dioxin-like CB was below the limit of detection, the TCDD TEQ value for that specific congener was not used in the calculation. The TCDD TEQs were reported as pg/g wet weight.

Statistics

PCB congener and DDT metabolite concentrations were compared on a wet weight basis (w.w.) and lipid weight basis (l.w.) as noted. Results were grouped and compared according to the relative age of the pup's dam (young, presumably primiparous versus old, multiparous). Paired *t*-tests or Student's *t*-tests were used to detect differences

between the perinatal capture and recapture and by dam age, where applicable, when data distribution was normal and variances were equal. For non-parametric data, a Mann-Whitney Rank Sum Test was used. Differences were considered significant when *p* was below 0.05. Correlation coefficients were determined by Spearman Rank Order Correlation. Chi-square, Fisher exact or z tests were used to detect differences in frequency or proportions of samples above detection limits or positive responses as noted. Statistical analyses were performed using Sigma Stat 2.0® (SPSS, Chicago, IL). Results are reported as mean and standard deviation (SD) in tables and are represented as mean and standard error (SE) in figures except where noted.

3.2 Results

OC concentrations

Fifty neonates were captured, and toxicological analyses were conducted on blood samples from 48. Forty-four of the original 50 were recaptured, and 42 had chemical analyses conducted on blood samples. CBs-77, -81, -126, -156, -157, -169, -189, *o,p'*-DDD, *o,p'*-DDT, chlordane and hexachlorobenzene (HCB) were below the detection limits (BDL) of the assay (approximately 0.03 ng/g) in all blood samples for this part of the study.

Congeners -138 and -153/87 combined accounted for just less than half of the sum of the detected congeners in pup blood and did not vary significantly from perinatal capture (mean 45.6% ± 10.3, range 25.9% to 93.5%, $n = 44$) to recapture (mean 44.5% ± 7.7, range 17.8 % to 63.6%, $n = 40$). Instead, CB-101/99/149/196 was typically the predominate congener group by percent of the sum PCBs at both time points (perinatal

capture mean 28.8% ± 10.8, range 6.0% to 59.0%; recapture mean 27.7% ± 11.6, range 14.4% to 75.1%).

Significant differences (by paired *t*-tests) were detected between contaminant concentrations in the blood of neonates and pups when data were separated by the age of the dam as depicted in Figures 3.1 and 3.2 (PCB congeners and DDT metabolites, respectively) and Table 3.1. Neonates of young dams had significantly higher w.w. concentrations of CB-101/99/149/196, -105, -118, -138,- 153/87, and *p,p'*-DDE in their blood than neonates of old dams. CB-170/194 and *p,p'*-DDD were detected only in the blood of neonates or pups of young dams. Three pups of old dams had detectable levels of *p,p'*-DDT and all the pups of young dams were below detection.

The change in congener concentration for individual seals over time was not calculated for concentrations of CB-128, -170/194, *p,p'*-DDT and *p,p'*-DDD in blood because so few samples (< 4 per group) had detectable levels. A Fisher exact test did not detect a significant difference in the number of samples above the detection limits (ADL) between groups. However, significant differences were found between pups of young versus old dams for concentrations of CB-101/99/149/196, -105, -138,- 153/87, and *p,p'*-DDE in blood. In each case, mean congener concentrations in blood of pups of young dams increased from perinatal capture to recapture while the mean change was negative in the pups of old dams (Figure 3.3). Lipid content did not have an effect on significance. The mean changes in *p,p'*-DDE followed the same trend and were 7.34 ± 10.8 ng/g for pups of young dams and -2.87 ± 5.0 ng/g for pups of old dams.

The concentration of only one congener, CB-153, showed a significant difference by sex of the pup (median of 22 female neonates = 1.3 ng/g w.w. versus median of 28 male neonates = 0.74 ng/g, Mann-Whitney Rank Sum test). CB-128 was above the detection limit in the blood of too few pups to analyze for significant differences in mean or median concentrations but it was detected in three females and only in one male. For the DDT metabolites, it is notable that three females had detectable levels of *p,p'*-DDD but all males were below detection.

Hemogram

The mean hematocrit significantly ($p < 0.001$) decreased from 43% in the neonates ($n = 50$) to 37% in the recaptured pups ($n = 43$). Total protein, total leukocyte, mature neutrophil, band neutrophil and monocyte counts did not differ significantly between neonates and pups. However, the neutrophil:lymphocyte ratio decreased significantly ($p < 0.001$) from neonates to pups (Table 3.2). When hemograms of neonates of young and old dams are compared (Table 3.3) the mean hematocrit, total plasma protein and absolute eosinophil counts were significantly greater in the neonates of old dams.

Total leukocyte counts were not significantly different between male and female neonates, however, recaptured male pups had significantly lower leukocyte counts than females (mean of 14,275 cells/µl versus 17,152, $n = 22$ and 19 respectively, Student's *t*-test). There were no significant differences in leukocyte types except the mean neutrophil count was lower in male pups (8,914 versus 11,843 cells/µl).

Hematologic values were examined for indications of infectious or inflammatory conditions that might be correlated to contaminant concentrations in blood. In neonates, increased immature (band) neutrophil counts were correlated to higher TCDD TEQs (correlation coefficient 0.347, $p = 0.016$, $n = 48$). In recaptured pups, the total leukocyte counts were positively correlated to higher total PCB concentrations in blood (correlation coefficient 0.359, $p = 0.023$, $n = 41$)

Haptoglobin

Levels of serum haptoglobin were examined as another potential measure of subclinical (grossly undetected) inflammation. Haptoglobin levels were compared with hemogram values to confirm an expected correlation with indicators of an inflammatory leukogram. Haptoglobin levels at recapture were correlated with the hematocrit (correlation coefficient of -0.588, $p < 0.05$), total protein (0.332), total leukocytes (0.337) and band neutrophils (0.452).

Haptoglobin levels were compared between the 48 neonates and the 43 recaptured pups. The mean values were 30 ± 45 (range 0 to 152) mg of hemoglobin-binding capacity per 100 ml of serum for neonatal samples and 82 ± 65 (range 0 to 194) for the recaptured pups. The recaptured pups had significantly higher levels as compared to the neonates (Mann-Whitney Rank Sum test, $p < 0.001$). Haptoglobin levels of recaptured pups were not significantly different from the mean 120 ± 50 of 18 adults (periparturient dams) that were concurrently tested. The neonate and pup haptoglobin levels were compared separately according to the relative age of the dam (Figure 3.4). There were no differences between neonatal and recapture levels for the pups of old dams, but

haptoglobin levels increased significantly from neonatal to recapture for pups of young dams (Mann-Whitney Rank Sum test, $p < 0.001$). Among neonate and older pups, there were no significant differences detected between the haptoglobin levels in pups of young dams and pups of old dams at either capture time. There were no significant differences between the mean haptoglobin levels in the adults and the pups of young dams, but levels in the pups of old dams were significantly lower than adults. No differences were detected that could be attributed to the sex of the pup. No direct correlations of serum haptoglobin concentration with blood OC concentrations were detected.

Humoral Immunity - tetanus antibody response

The development of serum tetanus antibody levels (in response to vaccination at the perinatal capture) was determined from serum samples collected 29 - 51 days post-vaccination. Figure 3.5 shows the increase in antibody titer, a 2-fold or greater increase is considered the expected response. A higher proportion of the pups of old dams (9 of 22) responded better than pups of young dams (1 of 21). The difference in the proportion of pups responding by the dams age was significant ($z = 2.443$, $p = 0.015$). However, the blood OC levels in the young dam's pup that responded well were not significantly lower than the rest of the cohort.

Total Immunoglobulins

Total immunoglobulin (Ig) concentrations did not vary significantly from perinatal capture to recapture when all non-moribund pups were considered. However, when the pups are grouped by the age of the dam, neonates of young dams had significantly lower Ig levels than neonates of old dams (mean 3.14 ± 1.2 mg/ml versus

4.6 ± 1.1 mg/ml, $p < 0.001$). Figure 3.6 shows the Ig concentrations in neonates and at recapture by the age of the dam. Immunoglobulin concentrations in pups of old dams did not differ significantly from perinatal capture to recapture (mean 4.6 ± 1.2 mg/ml and 4.5 ± 1.3 mg/ml, respectively). There were no differences between Ig concentrations between pups of young and old dams at recapture. Pups of young dams had a mean increase in Ig concentrations from perinatal capture to recapture of 1.0 mg/ml which was significantly greater than the increase of 0.1 mg/ml for pups of old dams (Mann-Whitney Rank Sum test, $p = 0.019$). The Ig concentrations in serum of pups of young dams increased from the perinatal level in 12 and decreased in 9 individuals. Among pups of old dams, 6 had an increase in concentration and 16 decreased. These changes were not significantly different between pups of young and old dams (Fisher Exact test).

Mitogen-induced lymphoproliferation responses

Lymphoproliferative responses of PBML paired samples to mitogen stimulation from both neonate and recapture samples were available from seven individuals. When results from 1995 and 1996 neonates were combined, the distribution of responses was normal.

Figure 3.7 shows the range of lymphoproliferative responses (as a stimulation index or SI) to two different concentrations of three mitogens (ConA, PWM and PHA). ConA at the lower concentration (0.5 µg/ml) yielded the optimal proliferation responses. Figure 3.8 documents the poor to moderate responses in the older (recaptured) pups compared to the neonates. When compared to the few adults and subadult samples

available from 1995, only one recaptured pup achieved a good lymphoproliferative response.

Neonate PBML responses (SI) from 1996 (n = 13) to the low dose ConA were negatively correlated ($p < 0.05$) to blood concentrations of CB-101/99/149/196 and –153/87 w.w. (correlation coefficients of -0.62 and –0.55, respectively). When 1995 and 1996 perinatal capture results were combined (n = 31) then CBs-101/99/149/196, -105, -118, and total PCBs w.w. were significant (correlation coefficients of -0.44, -0.85, -0.43 and –0.59, respectively). Additionally, the high dose ConA lymphoproliferative responses of PBML were negatively correlated with the lipid normalized concentrations of CB-105 and total PCBs (correlation coefficients of -0.81 and –0.36, respectively).

3.3 Discussion

Northern fur seals born to young (primiparous) dams have higher blood levels of PCB congeners than pups born to multiparous dams. These levels reflect exposure to high levels of PCBs in the milk they ingest (Beckmen et al., in press). Over the course of the nursing period, the first-born pups tend to have increases in blood OC concentrations, whereas pups of older dams, who start out with lower blood OC concentrations, tend to show decreases in blood OCs. The higher concentrations in the first-born were consistent with other marine mammal tissue residue studies but the decline over nursing (as seen in the pups of the old dams) had not been previously observed. The concentrations of *p,p'*-DDE were found at even higher concentrations than PCBs in the blood, especially in the neonates.

The first-born pups demonstrated poorer humoral immune responses to tetanus vaccination, lower immunoglobulin levels as neonates and increased haptoglobin levels midway through the nursing period compared to pups born to old dams. Among all neonates, regardless of dam age, poor lymphoproliferation responses to mitogen stimulation were correlated with higher blood levels of PCB congeners. Lymphoproliferative responses waned in the recaptured pups regardless of dam age or OC levels in blood. These results differed from previous observations in harbor seal pups. Northern fur seal pup PBML appeared to proliferate in response to ConA but at a level that was lower than the response of dam and subadult PBML tested. Ross et al. (1993; 1994) characterized the developmental aspects of the immune system of newborn harbor seals utilizing free-ranging dam-pup pairs. In the latter paper, the investigators describe mitogen-induced responses in nine pups, and they found that the PBML responded stronger than dam PBML to ConA, PHA and PWM. It was concluded that the strong proliferative responses of PBML to both T- and B-lymphocyte stimulating mitogens in newborn harbor seals indicated immunocompetence at that early age (Ross et al., 1994).

Typically, ConA and PHA elicit specific T-lymphocyte responses in mammals, while PWM elicits a mixed T- and B- cell response. This apparently holds true in harbor seals and beluga whales (*Delphinapterus leucas)*, the most extensively investigated marine mammals (De Swart et al., 1993; Dimolfetto-Landon et al., 1995; De Guise et al., 1996). However, in bottlenose dolphins ConA and PHA stimulate different lymphocyte subpopulations and are, therefore, not T-lymphocyte specific (Erickson et al., 1995).

Northern fur seal pups appear to be similar to dogs (*Canis familaris*) in that the lymphocyte responses to PWM and PHA are significantly lower at birth than adults (Gerber and Brown, 1974; Krakowka and Koestner, 1976). However, only a few adult and subadult fur seal PBML samples were available for stimulation with PHA and PWM in a preliminary study (data not shown). PHA and PWM induced minimal lymphoproliferative responses in subadult and adult northern fur seals. The lack of response in the pups noted with PHA as a mitogen may be simply due to lack of an adequate number of samples tested at the higher concentration. It seems unlikely that B-lymphocytes would lack the ability to respond and instead the mitogens may be less cell type-specific in fur seals.

Correlation coefficients of lymphoproliferative responses and blood PCB concentrations, though statistically significant, must be viewed with caution. A few individuals with very low to undetectable PCB congener concentrations also were in the upper ranges of lymphoproliferative responses. Additionally, a few individuals with detectable levels of CB-105 (ten) and CB-170 (three) had poor lymphoproliferative responses and the rank correlations were strongly influenced by these individuals. Thus, it is difficult to be certain that the elevated PCB levels in the blood negatively influenced the lymphoproliferative responses.

The newborn fur seal lymphocyte responsiveness and the comparative lack of lymphoproliferative response in older pups to polyclonal mitogen stimulation is difficult to explain. Cavagnolo (1979) found that the fur seal thymus involutes at about the time of weaning but is regenerated later (*sic*). He suggests that the stress of weaning and the

inhospitable rookery conditions may bring about a temporary immune suppression. It is possible a stress-induced immune suppression occurred 1-2 months into nursing in this study but the effects of neonatal OC exposure can not be ruled out as having an influence. Nor can the effects of prior handling of the pups and vaccination be eliminated. Mitogen proliferation assay or cryopreservation techniques are not likely sources of the observed differences since no changes in techniques, equipment, or stock reagents were made during 1996.

The parity of the dam in this study clearly affected neonatal serum Ig levels, probably through lower colostral transfer than from old, multiparous dams. Cavagnolo and Vedros (1979) found only a small amount of IgG (five percent of maternal level) and IgM in northern fur seal fetuses, indicating minor transplacental transfer (as seen in the dog) or fetal production. Their findings suggested that colostrum was the primary source of immunoglobulin (IgA was the highest) for a newborn fur seal but even at one week of age, the serum Ig levels were still low, being only slightly greater than at birth levels (Cavagnolo and Vedros, 1979). IgG slowly increased to 39% of the adult levels by 4 months of age (weaning). IgM rose linearly so that by 5 weeks it was 70% of the adult value. They suggested that IgM and non-specific cellular immunity must protect the pup from coliform bacteria and other pathogens encountered on the rookery. In the present study, immunoglobulin classes were not separated, but total Ig only increased significantly over neonatal levels in the pups of young dams which may be an indication of a higher rate of secondary infections (total leukocyte counts were also positively correlated with total PCB concentrations).

Northern fur seal neonates in this study were capable of developing antibodies when stimulated by vaccination with a specific antigen. Controlled studies in rabbits have provided evidence that PCBs alter humoral immunity (Koller and Thipen, 1973). Some PCB mixtures have a more profound influence on antibody-mediated immunity than cell-mediated immunity (Silkworth and Loose, 1981). The production of specific antibody in serum in response to introduction of an antigen can be used as a measure of the functional status of all three developmental phases (recognition, activation and expression) of the humoral immune response. Only 1 of the 21 pups of young dams, had a 2-fold or greater increase in anti-tetanus antibody. A significantly greater proportion (41%) of the 22 pups of old dams had a 2-fold or greater increase in antibody. Fewer of the pups of young dams responded well to this antigen challenge but there was not direct correlation between blood OC concentration and antibody titer. Anti-tetanus antibody production (IgM and IgG) is T lymphocyte dependent (Willcox, 1975). Guinea pigs fed commercial mixtures of PCBs at 0, 10, 50 or 250 ppm in the diet for eight weeks and given one to two doses of tetanus toxoid showed anti-toxin titers were inversely related to exposure dose (Vos and Van Driel-Grootenhuis, 1972). Both primary (IgM) and secondary (IgG) responses were suppressed at the 50 ppm level of exposure (Vos and Van Driel-Grootenhuis, 1972). An additional study in guinea pigs found decreased IgG production from tetanus toxoid injection after 3 weeks dietary exposure to 10 ppm of Aroclor 1260 compared unexposed controls (Vos and De Roij, 1972).

The immunotoxic effects of PCBs can be subtle and are not easily detected by routine clinical evaluation techniques (Bleavins and Aulerich, 1983). Typical gross

measures of the immune system (leukocyte counts, thymus and spleen weight or morphology) are not sensitive enough to detect effects at low dose, short-term exposures (Vos and De Roij, 1972; Street and Sharma, 1975). However, in a harbor seal study, De Swart et al. (1995b) found that leukocyte counts, neutrophils in particular, were higher in the higher exposure group especially during the second half of their 2½ year study. They speculate that the increased absolute neutrophil counts may be an indication of immunotoxicity and that either an increase in subclinical infection or an effect at the myeloid stem cell level (during bone marrow hematopoesis) was responsible. The present study did find a correlation between TCDD TEQ and band neutrophils in neonates and between total leukocytes and total PCBs in pups at recapture.

The northern fur seal neonate mean hematocrit was significantly higher (43.4%), compared to older pups (37.1%). This finding was consistent with values in free-ranging Steller sea lion (*Eumetopias jubatus*) pups (Rea et al., 1998) from the Gulf of Alaska. A mean hematocrit of 48.3% for Steller sea lion neonates declined to 32.8% in 4-week-old pups. The decline in hematocrit in the first few weeks after birth is a typical pattern in mammals that may be explained by plasma volume expansion with growth as well as short survival time of erythrocytes in neonates and conversion from fetal hemoglobin (Spensely et al., 1987). In a study of dietary OC exposure in harbor seals, higher hematocrits were seen in the higher OC exposure group but it was not considered to be due to the OC exposure since the difference decreased over time (De Swart et al., 1995b). In the present study, the finding of a slightly lower hematocrit in the higher exposure group of neonates contradicts the harbor seal findings but the harbor seals were older and

followed over a more prolonged period. The differences in the fur seals could be an indicator of another process such as anemia of inflammatory disease or better hydration. The former is unlikely to have developed over the relatively short observation period and the total plasma proteins were also lower.

Elevations of haptoglobin concentrations in serum or plasma have been demonstrated to be indicative of the acute phase response in laboratory and domestic animals as well as humans (Gruys et al., 1994). Haptoglobin elevations are induced by a variety of stressful physiological states including pregnancy, fever, neoplasia, inflammatory diseases and trauma. In Steller sea lions, Zenteno-Savin et al. (1997) documented a 3-fold increase in plasma haptoglobin levels in 1 to 10 week old pups from a population that had undergone severe declines compared to similarly aged pups from an increasing population. In the present study, the fur seal pups of young dams had significantly elevated mean haptoglobin compared to the pups of old dams but the difference was of a smaller magnitude (only 1/3 higher) than between two groups of Steller sea lions (Zenteno-Savin et al., 1997). In this study, there was a 3-fold increase in mean haptoglobin from the neonates to the four to eight week old pups of young dams. Whether an increase in haptoglobin is a biomarker of environmental contaminant exposure is highly speculative. It has been postulated that elevated mean haptoglobin levels in a subpopulation of river otters (*Lutra canadensis*) was due to oil exposure (Duffy et al., 1994).

When employing biomarkers as indicators of environmental contaminant exposure, it is critical to examine multiple biomarkers in an integrated fashion, since each

value could diverge from normal for a multitude of reasons besides contaminant exposure (Reijnders, 1994). Since environmental contaminants have specific mechanisms of toxicity they can influence antibody-mediated immunity while having no detectable effect on cell-mediated immunity. Therefore, no single assay of immune function is appropriate to detect chemically induced immune dysfunction (Silkworth and Loose, 1981). The suite of hematological and immunological assays employed here, suggest first-born northern fur seal pups with higher OC exposure have a decreased ability to produce antibodies to a specific antigen and may have an increased susceptibility to infectious organisms. Lymphoproliferative responses were negatively correlated with blood OC levels. These results appear consistent with the differential effects of OCs on lymphocyte subpopulations, as suggested by studies in different laboratory animal species (Neubert et al., 1992; Smialowicz et al., 1994). However, contaminant-induced immunosuppression in wildlife cannot be conclusively demonstrated without conducting controlled exposure trials on captive animals. A cause and effect relationship between chronic environmental contaminant exposure and a population decline may never be proven. Ross et al. (1996) concluded from the results of their studies that contaminant levels in harbor seals inhabiting polluted areas in North America and Europe are at risk of environmental contaminant-induced immunotoxicity. They speculate that this would result in diminished host resistance leading to increased incidence and severity of infectious disease in these populations. Although the present study was not designed to fully elucidate the role of OC contaminant exposure-induced immunosuppression in the decline of St. George Island northern fur seals, it did identify a cohort (first-born pups) that is at higher risk.

The potential effects of environmental contaminant exposure during a critical developmental life stage validates a reason for concern and emphasizes the need for further monitoring and research.

Table 3.1. Mean ± SD concentrations (number below detection) of PCB congeners in ng/g and TCDD toxic equivalency (TEQ) pg/g w.w. at perinatal capture and recapture 29 to 51 days later by relative dam age from St. George Island, Alaska, 1996.

Constituent	Neonates		Pups	
	of young dams n = 23	of old dams n = 25	of young dams n = 21	of old dams n = 21
CB101/99/149/196	1.23 ± 0.64 (1)*	0.98 ± 0.95	0.75 ± 0.20	1.06 ± 0.65
CB105	0.46 ± 0.21 (10)†	0.18 ± 0.04 (23)	0.30 ± 0.12 (10)	0.33 ± 0.17 (11)
CB118	1.09 ± 0.62 (2)*	0.67 ± 0.62 (3)	0.69 ± 0.19 (1)	0.78 ± 0.40 (4)
CB128	0.39 ± 0.09 (19)	< 0.04[a]	0.21 (19)	0.26 (19)
CB138	1.31 ± 1.69*	0.43 ± 0.23 (2)	0.55 ± 0.018	0.55 ± 0.30 (1)
CB153/87	1.95 ±1.17*	0.76 ± 0.47	1.04 ± 0.40	0.95 ± 0.47
CB170/194	0.39 ± 0.08 (20)	< 0.03[a]	< 0.03[a]	< 0.03[a]
CB180	0.47 ± 0.30 (10)†	0.26 ± 0.09 (16)	0.24 ± 0.12 (7)	0.33 ± 0.26 (14)
Total PCBs	22.85 ± 7.62*	18.53 ± 5.53	16.21 ± 4.54	19.11 ± 7.26
p,p'- DDE	12.97 ± 11.23*	2.94 ± 2.16	6.64 ± 3.42	5.90 ± 5.54
p,p'- DDD	1.06 ± 0.65 (20)*	< 0.2[a]	< 0.2[a]	< 0.2[a]
p,p'- DDT	0.69 ± 0.46 (19)	< 0.2[a]	< 0.2[a]	0.60 ± 0.50 (18)
TCDD TEQ	0.13 ± 0.10*	0.06 ± 0.06	0.08 ± 0.04	0.08 ± 0.06
blood % lipid	0.30 ± 0.39	0.22 ± 0.15	0.28 ± 0.48	0.31 ± 0.32

*significantly elevated than corresponding pups of old dams, $p < 0.01$

†also significantly increased when considered on a lipid weight basis

[a]all samples below detection limit

Table 3.2. Reference ranges of hemogram values in northern fur seals as neonates and 29 to 51 days later as pups from St.George Island, Alaska, 1996.

Constituent	Neonates	n = 50	Pups	n = 42
	Range	Mean ± SD	Range	Mean ± SD
Hematocrit (Hct) %	35 - 57	43 ± 5*	32 - 44	37 ± 3
Total protein g/dl	6.1 - 8.8	7.4 ± 0.6	6.1 - 8.6	7.1 ± 0.5
Total Leukocytes/µl	7425 - 20570	12615 ± 2921	7728 - 29115	13832 ± 3880
Neutrophils:Lymphocytes	1.1 - 14.5	4.7 ± 2.9*	0.8 - 7.5	3.0 ± 1.9
Neutrophils/µl	4217 - 17485	8211 ± 2536	4525 - 20672	9079 ± 3053
Band neutrophils/µl	0 - 894	295 ± 225	0 - 1165	240 ± 291
Lymphocytes/µl	802 - 4162	1954 ± 842	1258 - 7983	3227 ± 1487†
Monocytes/µl	189 - 3396	1288 ± 696	118 - 3785	1047 ± 665
Eosinophils/µl	0 - 2990	848 ± 628*	0 - 1113	221 ± 230
Basophils/µl	0	0	0	0

*****significantly elevated compared to pups, $p < 0.001$

†significantly elevated compared to neonates, $p < 0.001$

Table 3.3. Mean ± SD of hemogram constituents in neonatal northern fur seal pups compared by relative dam age from St. George Island, Alaska, 1996.

Constituent	Neonates	
	of young dams n = 22	of old dams n = 25
Hematocrit %	42 ± 4	45 ± 5*
Total Protein	7.1 ± 0.4	7.6 ± 0.6*
Total leukocytes/µl	13171 ± 3164	14421 ± 2912
Neutrophil:Lymphocyte	4.2 ± 2.2	5.6 ± 3.4
Neutrophils/µl	8262 ± 2508	9429 ± 2236
Band neutrophils/µl	345 ± 290	325 ± 233
Lymphocytes/µl	2425 ± 944	2111 ± 917
Monocytes/µl	1197 ± 793	1385 ± 654
Eosinophils/µl	757 ± 488	1251 ± 778*
Basophils/µl	0	0

*****significantly elevated over neonates of young dams, $p < 0.01$

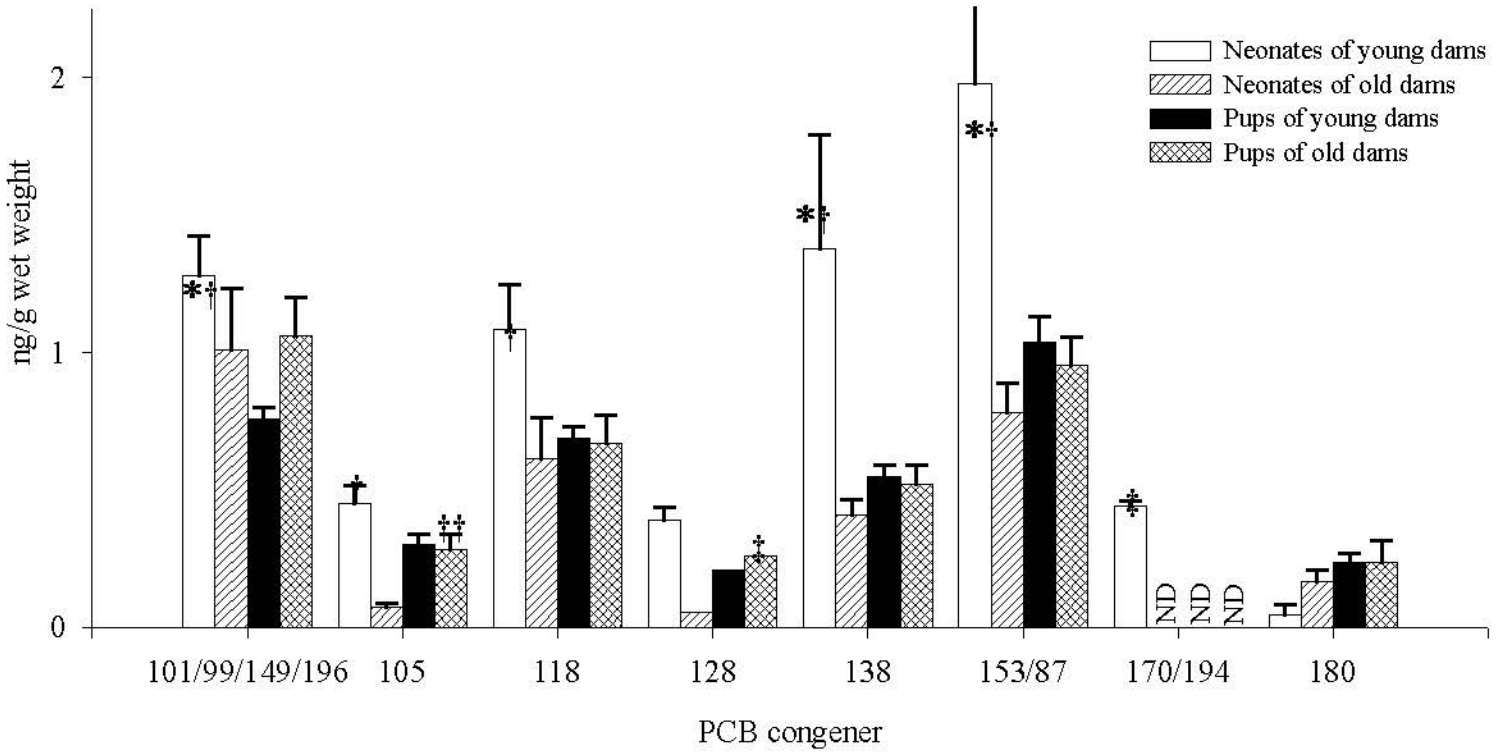

Figure 3.1. Mean ± SE PCB congener concentrations (ng/g wet weight) in northern fur seal blood from 21 pups of young dams and 21 pups of old dams sampled as neonates and at recapture 29 - 51 days later as pups on St. George Island, Alaska, 1996.

* significantly greater conentration in neonates than pups
† significantly greater concentration in the neonates of young dams than in the neonates of old dams
†† significantly greater in recaptured pups than neonates
‡ too few ADL to make statistical comparison
ND indicates all samples in group were below detection limits

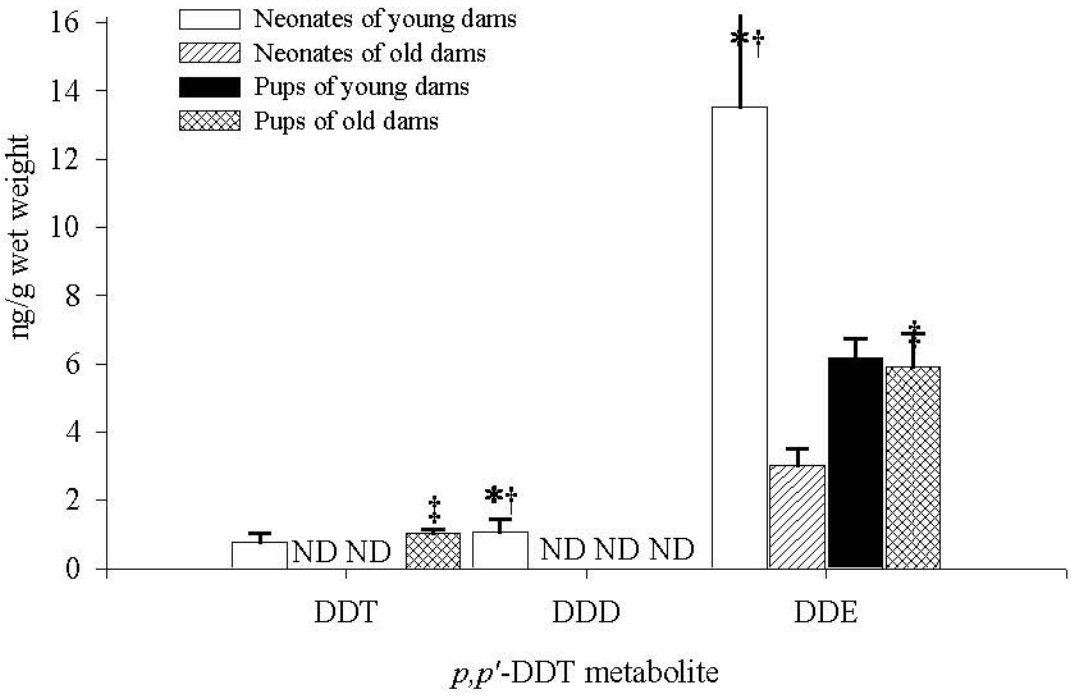

Figure 3.2. Mean ± SE DDT metabolite concentrations (ng/g wet weight) in northern fur seal blood from 21 pups of young dams and 21 pups of old dams sampled as neonates and 29 - 51 days later as pups on St. George Island, Alaska, 1996.

*significantly greater conentration in neonates of young dams than pups
significantly greater concentration in neonates of young dams than in neonates of old dams
‡significantly greater concentration in pups of old dams than as neonates
ND indicates all samples in group below detection limits

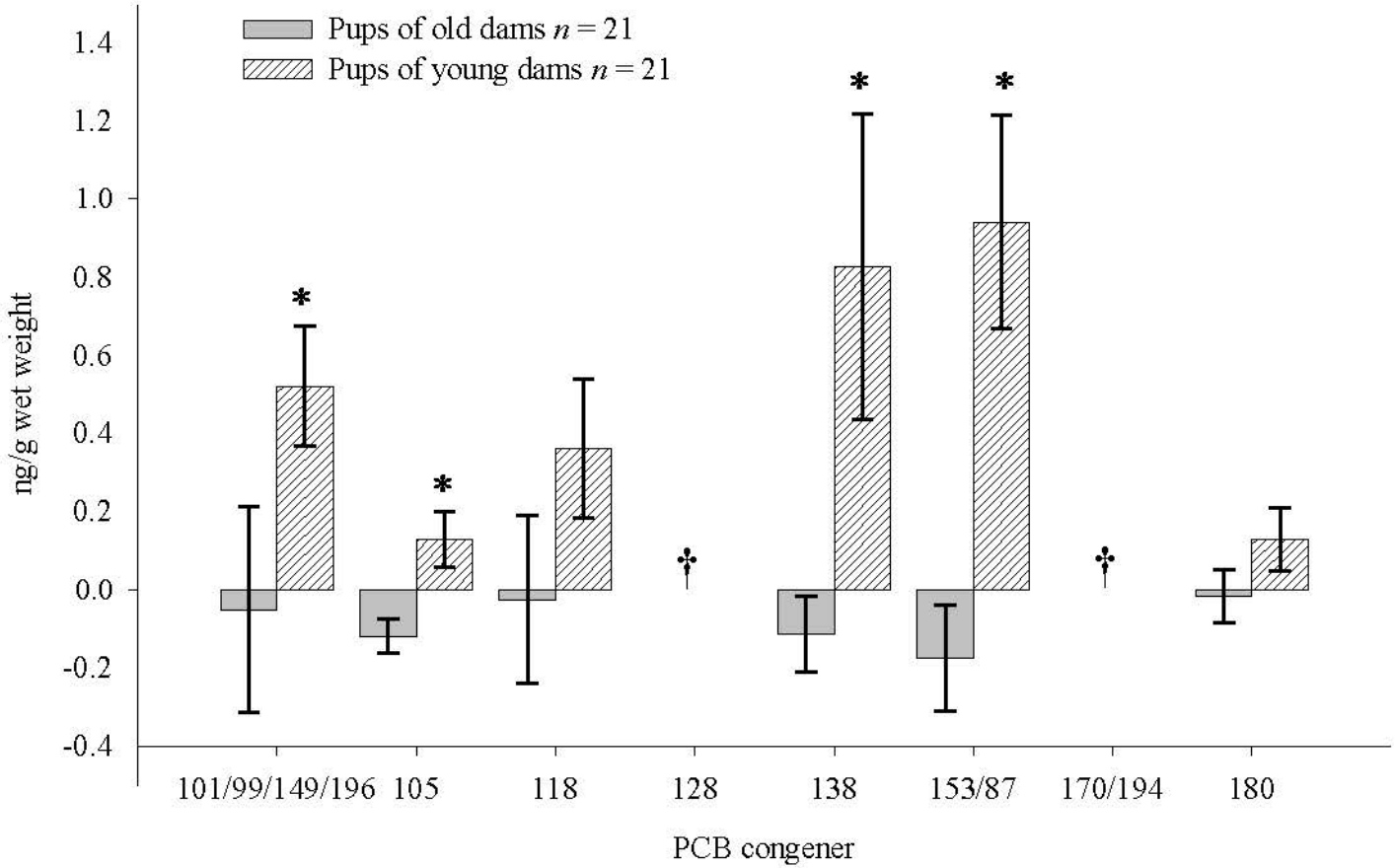

Figure 3.3. Mean ± SE differnce in PCB congener concentrations (ng/g wet weight) from perinatal blood sample to recapture blood sample 29 to 51 days later in northern fur seal pups grouped by relative age of dam on St. George Island, Alaska, 1996.

* significantly greater change in concentration than in pups of old dams

† change not depicted- only two samples above detection for pups of young dams and one pup of an old dam

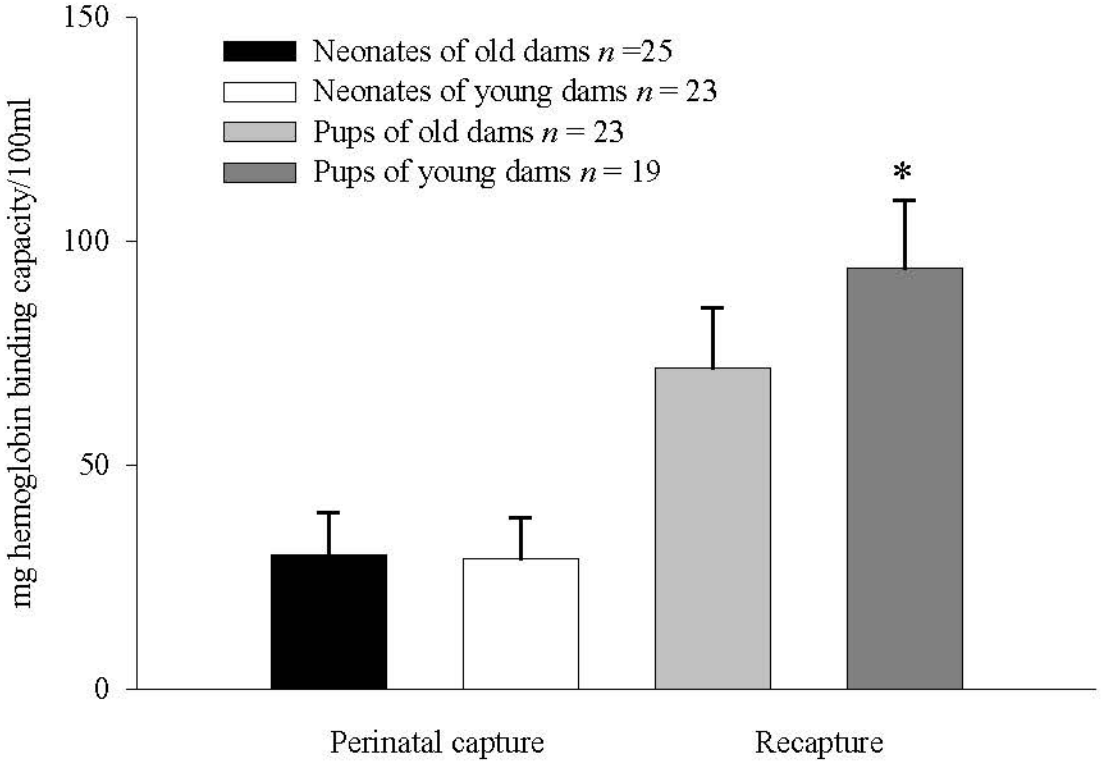

Figure 3.4. Mean ± SE haptoglobin levels (mg hemoglobin binding capacity/100ml of serum) in neonates and at recapture 29 to 51 days later from northern fur seal pups grouped by relative dam age on St. George Island, Alaska, 1996.
* significantly elevated over neonatal level ($p < 0.001$)

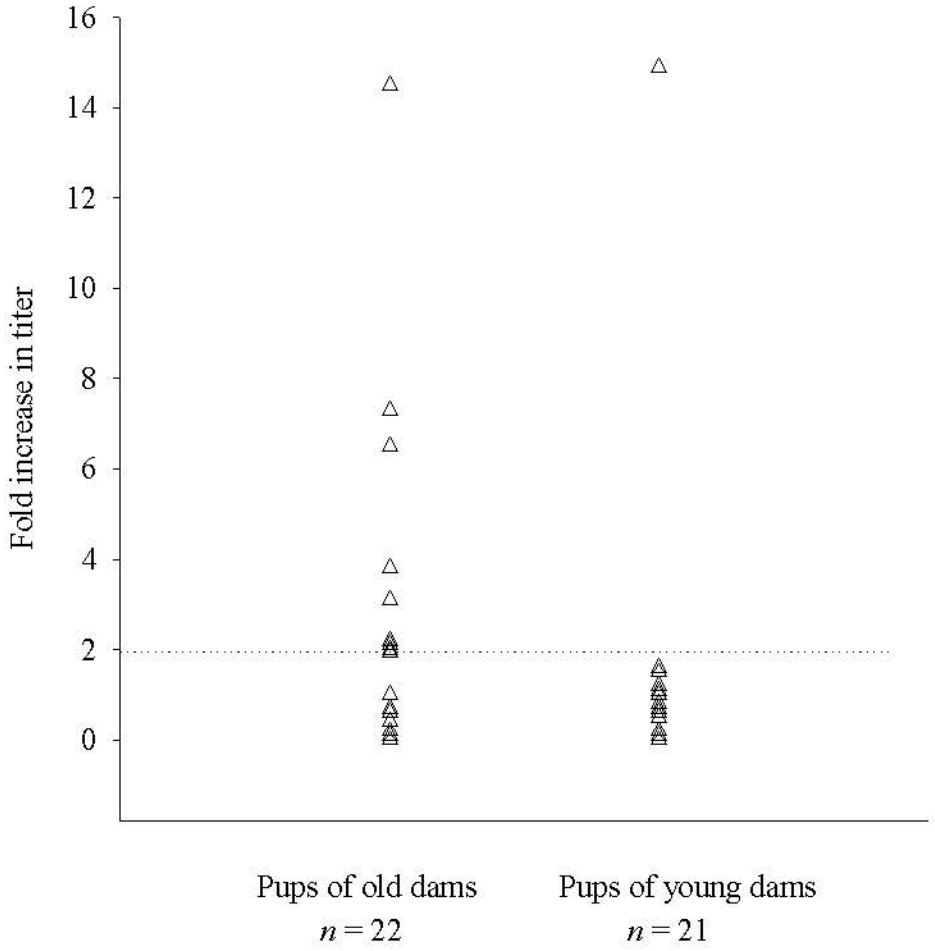

Figure 3.5. Change in serum tetanus antibody titer 29 to 51 days post-vaccination with tetanus toxoid antigen in 43 northern fur seal pups by dam age on St. George Island, Alaska, 1996.

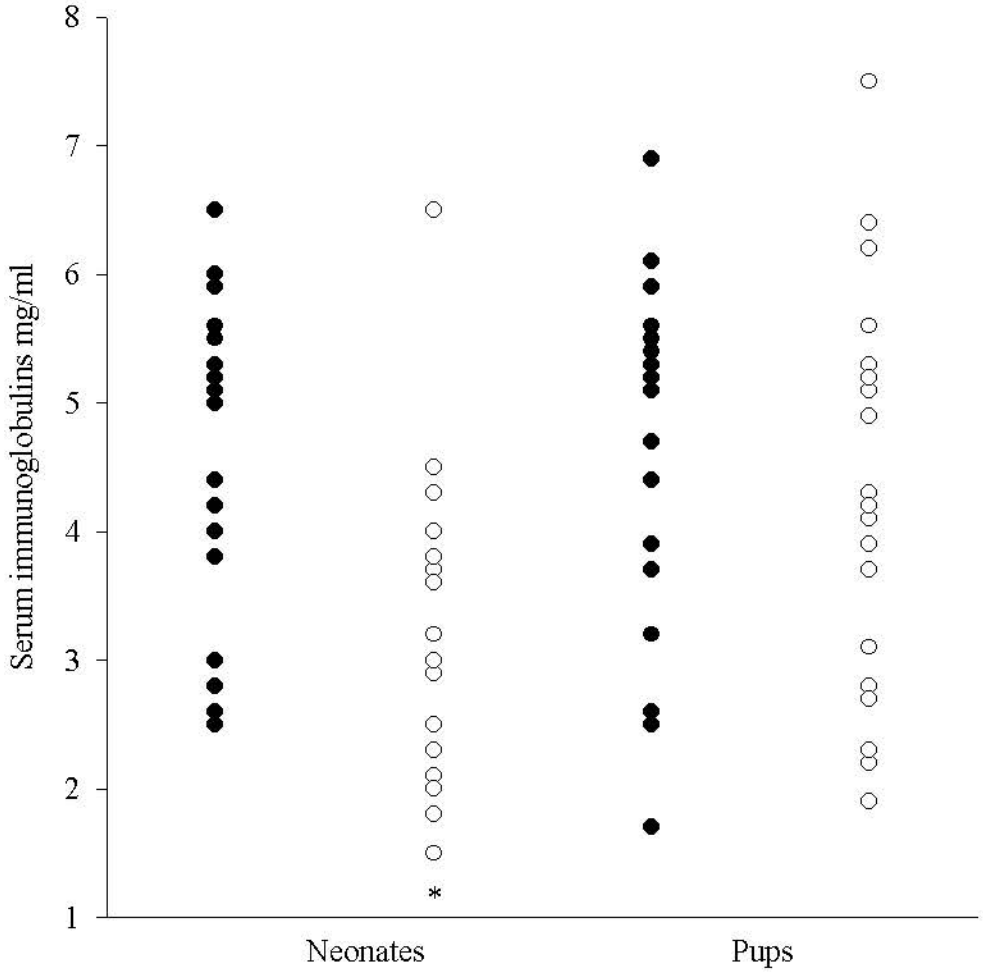

Fig 3.6. Serum concentrations of total immunoglobulins in northern fur seal pups as neonates and 29 to 51 days later (21pups of old dams indicated by closed circles, 21 pups of young dams indicated by open circles) from St. George Island, Alaska, 1996.

* mean concentration in neonates of young dams significantly ($p < 0.001$) lower than neonates of old dams

mean concentration in pups of young dams significantly greater than as neonates.

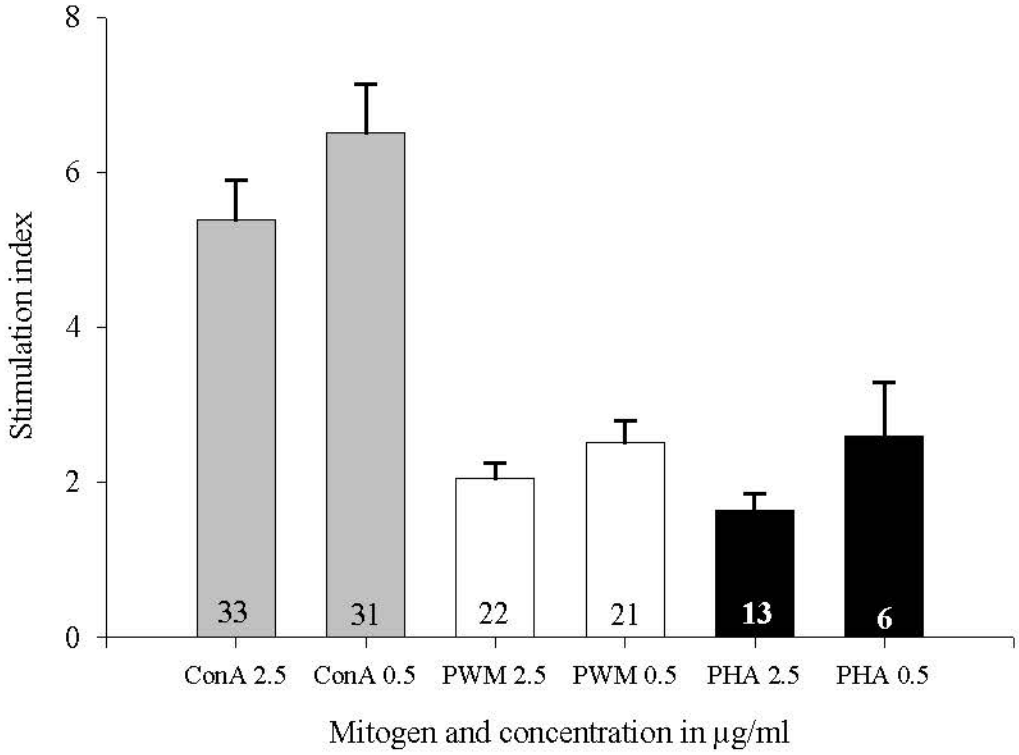

Figure 3.7. Mean ± SE lymphoproliferative responses of northern fur seal pup PBML cryopreserved in 1995 and 1996 on St. George Island, Alaska to three mitogens: concanavalin A (ConA, gray bars), pokeweed mitogen (PWM, white bars), and phytohemaglutinin (PHA, black bars), at concentrations of 0.5 and 2.5 µg/ml each, using a BrdU ELISA. Numbers within bars are sample sizes.

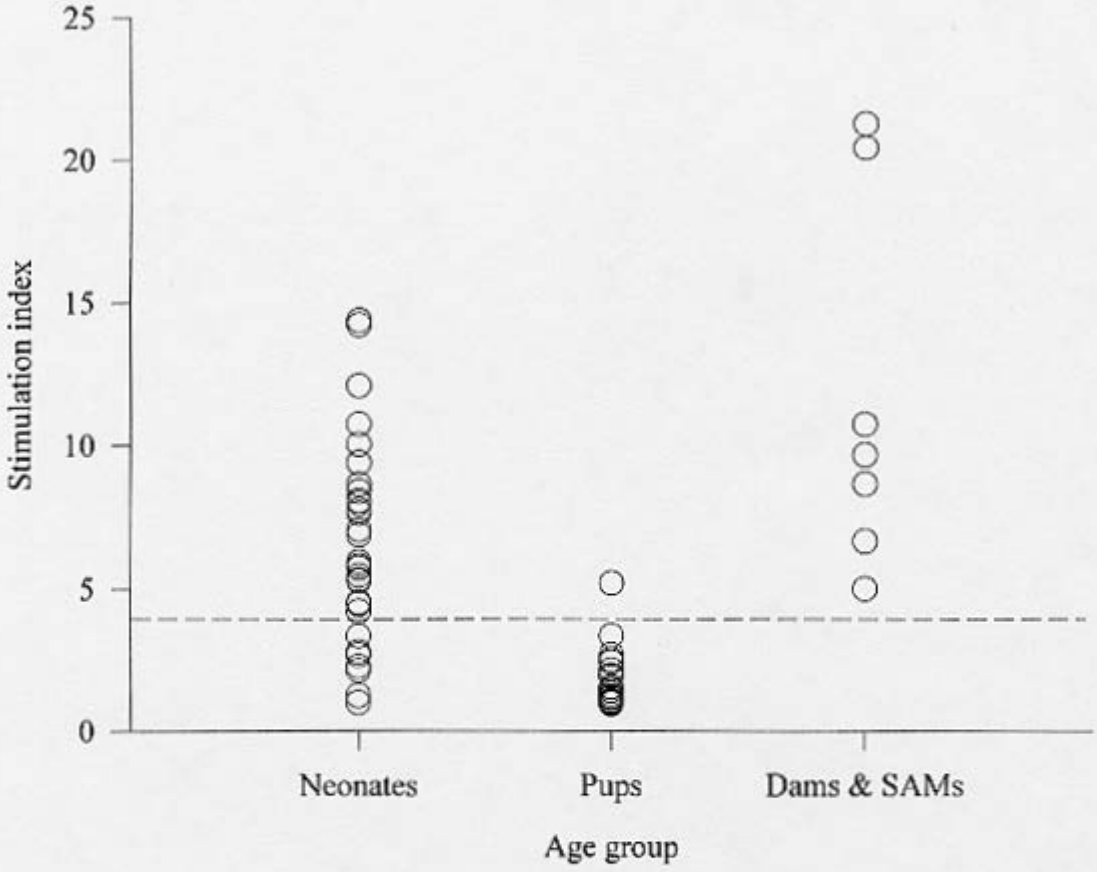

Figure 3.8. Lymphoproliferative responses by BrdU ELISA on previously cryopreserved northern fur seal lymphocytes from 31 neonates, 24 pups at 29 - 51 days post-perinatal capture, 4 dams and 3 sub-adult males (SAMs) to 0.5 ug/ml of ConA from St.George Island, Alaska, 1995 - 1996. SI below dashed line are considered poor to moderate lymphoproliferation responses.

Chapter 4

Association of Organochlorines in Milk and Pup Blood with Serum Retinol and Thyroxine Levels in Northern Fur Seal Pups (*Callorhinus ursinus*)

4.0 Introduction

Exposure to organochlorine (OC) contaminants such as polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-*p*-dioxins (PCDDs), may affect levels of serum retinol (vitamin A) and thyroid hormones in wild vertebrates. Brouwer et al.(1990) proposed that alterations in retinol and thyroid hormone metabolism may serve as sensitive indicators of exposure to certain PCBs and adverse effects in wild vertebrates.

Depressed serum retinol and thyroid hormone levels may be directly involved in the manifestation of PCB toxicity (Brouwer, 1991). Both thyroid hormone and retinol are critical to the development and maintenance of homeostasis in mammals. Immune system function and differentiation, as well as growth and maintenance of epithelial tissue are some important processes affected by retinol levels (Dennert, 1984). Contaminant-induced thyroid hormone depletion has serious consequences because of the critical role of thyroxine in metabolism, growth and development. Neurological development can also be seriously disturbed with either excessive or deficient thyroid hormone levels.

Laboratory rodents exposed to PCB congeners, such as CB-77, experienced a decline in both circulating thyroid hormones and retinol levels (Brouwer et al., 1986b; Brouwer et al., 1988; Durham and Brouwer, 1990). The decline in serum thyroid hormones occurs in a dose-dependent fashion in rats exposed orally to PCBs by gavage

and in rat pups born to dams eating contaminated feed during gestation and lactation (Byrne et al., 1987; Goldey et al., 1995). In addition to rodent studies, mink (*Mustela vison*) in laboratory studies experienced reduced retinol concentrations in liver and lung tissue following the oral administration of complex technical OC mixtures (Håkansson et al., 1992).

Captive juvenile harbor seals (*Phoca vitulina*) fed naturally OC-contaminated fish from the Baltic Sea showed a correlation between increased OC dose and reduced retinol levels in plasma (De Swart et al., 1994). Similarly, adult female harbor seals fed OC-contaminated fish from the Wadden Sea had depressed plasma retinol and thyroid hormone levels compared to seals fed less contaminated fish (Reijnders, 1986). Furthermore, the OC-induced plasma retinol depression was resolved with subsequent feeding of less contaminated fish from the Atlantic Ocean. Studies in free-ranging marine mammals have correlated lowered circulating levels of retinol and thyroid hormones with increased environmental exposure to OCs (Jenssen et al., 1995; Beckmen et al., 1997).

The effects of PCB and PCDD exposure on circulating and hepatic retinol levels cannot be fully explained by a single mechanism. Species differences occur, but, in general, PCBs and PCDDs alter critical steps of retinol metabolism (reviewed in Zile, 1992). Brouwer and van den Berg (1986a) and Brouwer et al. (1986b; 1988) demonstrated in rats and mice, an interference of the formation of the transthyretin (TTR)-retinol binding protein (RBP) complex occurring in association with exposure to metabolites of the PCB congener, CB-77. There is displacement of thyroxine (T4) from

the thyroxine-binding site on TTR by the hydroxy metabolites of CB-77, resulting in loss of the new free T4 from the circulation by rapid transport into tissues and enhanced renal excretion (Brouwer and van den Berg, 1986a). Investigators speculate that the interaction leads to a conformational change that disrupts retinol-RBP binding to TTR and unbound retinol-RBP passes across the glomerular membrane, enhancing excretion and leading to a decline in plasma levels (Brouwer et al., 1988). This type of interaction, in which an environmental contaminant blocks a hormone receptor or interferes with the hormone transport/binding process, describes an "endocrine disrupting chemical."

This study determined retinol and thyroid hormone levels in serum samples obtained from northern fur seal pups on the rookeries of St. George Island, Alaska during a field investigation of potential adverse effects of pre-weaning OC exposure. Whole blood concentrations of selected PCB congeners were measured in neonates and again in the same individual pups four to seven weeks later in the 4-month long nursing period. Concentrations of serum retinol were determined from matched archived serum samples. Free and bound serum thyroxine and triiodothyronine levels were also determined from the latter capture. The effect of age and sex of the pup on serum retinol and thyroid hormone levels was evaluated. Correlations between retinol, thyroid hormones and PCB congener concentrations were determined.

4.1 Materials and Methods

Study site

Northern fur seals were captured on four of the six rookery beaches of St. George Island, Alaska (N56° 34' W169° 41'), one of the two main breeding islands in the

Pribilof group in the Bering Sea. The treeless, wind-swept sub-arctic island is located 31 km from the continental shelf break. The rookery beaches are composed of sand, gravel and boulders of volcanic origin. During the fur seal occupation from May to November, the weather is typically overcast and marked by rain, fog with mean air and water temperatures around 5°C.

Animal criteria, capture, and marking techniques

Northern fur seal neonates were captured between 17 July – 5 August 1995 (n = 20) and 13 July – 3 August 1996 (n = 50), with a noose pole extended from a 3-person roving 'blind box' constructed of plywood (approximately 1x1.5x2m) using methods detailed elsewhere (Antonelis, 1992; Boltnev et al., 1998). This 'blind box' afforded protection of personnel from aggressive territorial bulls and allowed access to most of the rookery with minimal animal disturbance.

All neonates were accompanied by their dams at the initial capture and dams were classified as young ($\leq$5 years of age and likely to be primiparous) or old (>7 years, assumed to be multiparous) by body size, pelage and vibrissae characteristics (Vladimirov and Nikulin, 1993). Dams of a subset of neonates (11 in 1995, 22 in 1996) were captured at the same time for blood and milk sampling. The vast majority (all but two) of the 1996 neonates included in the study were estimated to be less than seven days old, based on the condition of the umbilical cord remnant or degree of umbilical healing, neonatal behavior and observation of characteristic periparturient/pre-estrus behavior of the dam (Gentry, 1998). Although neonates were observed in contact with their dams

when selected for capture, the timing, duration and volume of their most recent suckling bout was not known.

Prior to release, blood was collected in both years as described below. Neonates were marked with unique symbols by clipping the guard hairs on the top of the head and then applying gel hair bleach (Clairol Beyond Blond®).

A cohort of 44 four- to eight-week-old pups of the 1996 group was recaptured at intervals ranging from 29 to 51 days later (mean 39 ± 6) for repeat blood collection (between 24 August and 12 September 1996). Pups were recaptured either individually using stealth and a net or by herding all pups on a section of the rookery.

Blood sample collection and measurements

Seals were physically restrained for blood collection in a neoprene vest secured with Velcro® tabs, resting in ventral recumbancy on a V-shaped plywood platform. Blood samples were obtained from a superficial plantar flipper vein (2 samples were drawn from the caudal gluteal vein via syringe) using a 21 gauge Venoset® blood collection set into evacuated tubes: Venoject II® 5 ml plastic tubes containing dry EDTA as an anticoagulant (for OC concentrations), and Terumo® serum separator tubes (both by Terumo Medical Corp. Elkton MD) for the harvest of serum. After gentle mixing, 5 ml of EDTA whole blood was transferred to a methylene chloride-rinsed glass vial with a Teflon lined lid for OC analysis and frozen (to less than -20°C) within 6 hours of collection. Serum was obtained after clotting and centrifugation of a Terumo® serum separator tube. The harvested serum was transferred to cryogenic vials and frozen to -

70°C within 6 hours of collection until thawed for the retinol and thyroid assays described below.

The mass of each pup was determined by placing the pup in a burlap sack or bucket and suspending it from a spring scale. The mass, minus the tare mass, was recorded to the nearest 0.2 kg. Standard length was measured to the nearest 0.5 cm from the tip of the nose to the tip of the tail with the pup restrained in ventral recumbancy. Girth was measured under the foreflippers at the level of the axilla and recorded to the nearest 0.2 cm during exhalation.

Dams captured for milk samples were manually restrained and injected with 5 IU oxytocin intramuscularly. After five minutes, a minimum of 5 ml of milk was manually expressed by digital massage directly into a methylene chloride rinsed vial. Milk samples were frozen to -20°C until analysis. Blood samples were obtained and handled in a similar fashion as in the pups except CPT™ tubes were not used.

Capture and sampling activities were conducted under Marine Mammal Protection Act/Fur Seal Act permits #837 or #1003 and a research protocol approved by the Institutional Animal Care and Use Committee of the University of Alaska Fairbanks.

Thyroid hormones

Serum thyroid hormone levels were determined at the Animal Health Diagnostic Laboratory at Michigan State University, East Lansing MI. Thyroid hormone analyses were conducted as follows: total thyroxine (TT_4) by a radioimmunoassay (RIA) kit (Chiron Diagnostic Corp. East Walpole MA); total triiodothyronine (TT_3) by in-house RIA, charcoal separation method as described by Refsal et al. (1984); unbound thyroxine

(Free T_4 or FT_4) by direct serum analog RIA (Chiron Diagnostic Corp); unbound triiodothyronine (Free T_3 or FT_3) by direct serum analog assay (Chiron Diagnostic Corp.). Total T4 and TT3 are reported in nmol/L, and FT4 and FT3 in pmol/L of serum. Due to limitations on the amount of serum that could be obtained from the neonates, thyroid hormone analysis was performed on the recaptured pup samples only.

Retinol

Retinol (vitamin A) analyses were conducted by the Animal Health Diagnostic Laboratory at Michigan State University. The level of retinol in serum was determined by high-performance liquid chromatography (HPLC) after alcohol/hexane extraction utilizing the methods of Stowe (1982) with the modifications described by Dennison and Kirk (1979). Retinol concentration in serum was determined in serum samples collected at the perinatal capture and at recapture 29 to 51 days later and reported in mg/ml.

Analytical techniques for organochlorine contaminants

Concentrations of eleven dioxin-like congeners (CBs -77, -81, -105, -118, -126, -156, -157, -169, -170, -180 and -189) were resolved from other selected CBs (CBs -101, -128, -138, and -153) and chlorinated hydrocarbons (e.g. *p,p*'-DDD, *p,p*'DDE, *p,p'*-DDT) in whole blood or milk using a rapid HPLC coupled with a photodiode array detection method (Krahn et al., 1994). Analytical techniques for the OC determination, lipid determination and calculation of values are described in detail in Chapter 2 and Beckmen, et al. (in press).

Statistics

Results were grouped and compared according to the pup's sex and age. Paired *t*-tests or Student's *t*-tests were used to detect differences between perinatal and recapture pups and between sex, as applicable, when data distribution was normal and variances were equal. For non-parametric data, a Mann-Whitney Rank Sum Test or Wilcoxon Signed Rank Test was used. Significant level was set at $p < 0.05$. Spearman Rank Order Correlation Coefficients were calculated using PCB congeners on a wet weight (w.w.) basis for blood and lipid weight (l.w.) basis for milk as noted. A sequential Bonferroni test was used when multiple correlations were tested to determine a table-wide α level of significance (Rice, 1989). Thyroid concentrations were log transformed for linear regression with PCB congener concentrations. Statistical analyses were performed using Sigma Stat 2.0® (SPSS, Chicago, IL). Results are reported as mean and standard deviation (SD) in tables and are reported as mean and standard error (SE) in figures except where noted.

4.2 Results

Retinol levels

Serum retinol concentrations were determined for neonates in both 1995 ($n = 20$) and 1996 ($n = 43$). Mean concentrations were significantly ($p < 0.001$) higher in the 1995 samples (343.2 ± 74.8 mg/ml) versus 1996 (251.9 ± 104.6 mg/ml). Mean concentrations of retinol in recaptured pups in 1996 were more than twice ($p < 0.001$) their neonatal levels (251.9±105.9 mg/ml in neonates with a range of 64 - 561 mg/ml versus 551.8 ± 201.8 mg/ml recaptured pups with a range of 226 - 1119 mg/ml, $n = 43$). No significant correlations between days from previous capture and retinol levels or increase in retinol

level were detected. Body mass was correlated with retinol levels for neonates (Spearman rank correlation coefficient 0.371, $p = 0.015$), but not for each gender examined separately. No statistically significant differences in retinol levels were detected between male and female pups, however, when mass is plotted against retinol level, there is a trend for females to have slightly higher retinol levels than the heavier males (Fig. 4.1).

Thyroid hormones

Serum thyroid hormones were determined for recaptured pups only. TT4 and FT4 were more highly correlated than TT3 and FT3 (Fig. 4.2). Table 4.1 gives the mean and SD values for the various serum thyroid hormones and ratios by sex (one moribund, septicemic pup was included only in the regression analysis). Female pups had significantly higher mean TT4 and FT3 than male pups (Fig. 4.3). TT4, FT4 and FT3 were strongly correlated with mass ($r = 0.496, 0.567, 0.602$, respectively, $p < 0.05$) only in female pups but not in males. There was no correlation between length and thyroid hormones.

Correlation with other hematological values and OC levels

Retinol levels in neonates were negatively correlated to two recalcitrant PCB congeners in whole blood: CB-138 and CB-153/87 (rank correlation coefficients of –0.403 and –0.452, respectively, $p < 0.05$). Serum retinol levels in neonates were also negatively correlated to the TCDD TEQ in the perinatal milk (correlation coefficient of -0.475, $p = 0.029$, $n = 21$). Refer to Tables 2.1 and 3.1 in previous chapters for mean PCB congener concentrations in milk and blood.

Serum retinol concentrations were significantly correlated with TT4, FT4 and TT3 (Spearman rank correlation coefficients = 0.543, 0.515 and 0.393, respectively, table wide $p < 0.05$) but not FT3. TT4 was negatively correlated with CB-101/99/149/196, CB-118, CB-138, and TCDD TEQ (Spearman rank correlation coefficients = -0.313, -0.519,

-0.457, -0.354, respectively, table-wide $p < 0.05$). Figure 4.4 shows the linear regression plot of the log transformed FT4 to the concentration of CB-118 w.w. in blood. The ratios of TT3:TT4 and TT4:FT4 were not correlated significantly with any of the PCB congeners detected in blood.

4.3 Discussion

The negative correlation of serum retinol and thyroid hormones levels with increasing concentrations of the PCB congeners in northern fur seal pup whole blood indicates a potential for use as biological markers of environmental contaminant exposure. This is similar to the findings of Jenssen et al. (1995) in gray seal (*Halichoerus grypus*) pups in Norway. They found a negative correlation between the sum of 22 PCB congeners in whole blood and plasma retinol levels. They concluded that retinol was a useful biomarker for assessing effects of low to moderate PCB exposure in gray seal pups. Although analytical differences make direct comparisons difficult, the mean sum of 22 PCB congeners in the whole blood of 27 gray seal pups was less than half the mean total PCBs in blood of the northern fur seal neonates in this study (9.10 ± 7.72 versus 20.7 ± 6.9 ng/g w.w.). Northern fur seal neonates, with the highest blood levels of PCBs,

had significantly lower retinol levels than older pups with low PCB levels but age alone may have accounted for this.

Thyroid hormone levels change dramatically in the first weeks of life in phocid seals (Englehardt and Ferguson, 1980; Little, 1991; Haulena et al., 1998; Hall et al., 1998; Woldstad and Jenssen, 1999). However, similar data are not available from any otariid species. Among northern fur seal pups, halfway through the 4-month nursing period, there was still a wide variation in thyroid hormone concentrations within a two to three week age range. Thus, in a group of neonatal pinnipeds or any species of wildlife, caution must be exercised when attempting to draw conclusions about a particular contaminant exposure effect based solely on thyroid hormone levels. Diurnal variation, molting, chronic disease and a host of other biological factors as well as contaminants can effect thyroid levels in mammals. Therefore, to be useful, baseline studies in the species of interest are necessary to understand the effects of these factors (Woldstad and Jenssen, 1999).

Hall et al. (1998), in their study of thyroxine as a biomarker of OC exposure in gray seals, failed to find any differences in thyroid hormone levels related to the sex of the pup. Northern fur seals pups show significantly higher TT4 and FT3 in females. Thyroid hormone values in northern fur seal pups were in a range similar to pre-weaning gray seals (Hall et al., 1998) (i.e.TT4 gray seal pups 62.2 ±3 6.1 nmol/L versus 67.2 ± 21.0 nmol/L in northern fur seal pups with sexes combined). In fur seals, TT4 and FT3 levels were significantly higher in female pups, which were also significantly smaller and lighter than males. Females were also smaller at birth and growth rates did not differ

between sexes (Chapter 5). Thyroid hormone levels were only correlated with mass in female pups. Jenssen et al. (1994) found mass was correlated with TT4 and FT4 in gray seals.

Jenssen et al. (1994) found that among 17 gray seal pups from Norway, those with the lowest plasma thyroxine levels had the highest PCB levels, but these were not significantly correlated (*sic*). In another gray seal study, investigators found a weak negative correlation between TT4:FT4 and the sum of PCBs in the blood (Jenssen et al., 1995). That study was criticized by Hall et al. (1998) because "TT4 levels are known to decrease with age and cumulative PCB exposure will inevitably rise". However, this is not the case in fur seals. Northern fur seal neonates have the highest blood OC levels, even higher than pups, subadult males or lactating females (Beckmen et al. in press). Of the neonates, those from young (primiparous) dams have the highest OC exposure. Additionally, Jenssen et al. (1994; 1995) measured blood (whole and packed red cell) concentrations of PCBs, not cumulative exposure over the entire nursing period.

In human infants, exposure to dioxins and PCBs (expressed as TEQs) in breast milk is correlated with increases in TSH and decreased plasma TT4 and FT4 two weeks after birth (Koopman-Esseboom et al., 1994). In northern fur seal pups a significant negative relationship between thyroid hormones and milk exposure was not detected, but there was a negative correlation between the dam's milk as TCDD TEQ and the neonate's serum retinol levels. Also, the northern fur seal pups with the highest retinol levels had the lowest blood PCB levels. The reduction in retinol levels in northern fur seal neonates with the highest milk exposure was significant but not quite as dramatic as the 45%

reduction in retinol seen by Brouwer et al. (1989) when highly PCB-contaminated fish from the Wadden Sea was fed to harbor seals for 1-2 years.

The negative correlation of retinol and thyroid hormones with PCBs in northern fur seal blood indicates that levels of these constituents may be reduced as a result of PCB exposure in young pups. Thus, PCB exposure in young pups has the potential to affect immune function, and therefore health, both through direct immunosuppression and indirectly by lowering retinol and thyroxine. There are no published reports demonstrating indirect immunosuppression by PCBs via this mechanism.

The wide range of values found in northern fur seal neonates and pups indicates that mean retinol or thyroxine levels for a small random group of fur seals would be inappropriate for a direct assessment of contaminant exposure for a population. Instead, multiple indicators of PCB exposure from individual neonates should be utilized in conjunction with relevant biological data such as gender, age, and birth order.

Table 4.1. Mean ± SD concentrations of thyroid hormones in serum of female and male northern fur seal pups from St. George Island, Alaska, 1996.

Constituent	Females $n = 20$	Males $n = 22$
TT4 nmol/L	74.0 ± 24.5*	59.4 ± 16.3
FT4 pmol/L	33.9 ± 10.0	28.7 ± 6.6
TT3 nmol/L	1.5 ± 0.6	1.3 ± 0.3
FT3 pmol/L	5.7 ± 1.7*	4.5 ± 1.0
TT3:TT4	0.020 ± 0.005	0.022 ± 0.004

* significantly greater than in male pups, $p < 0.05$

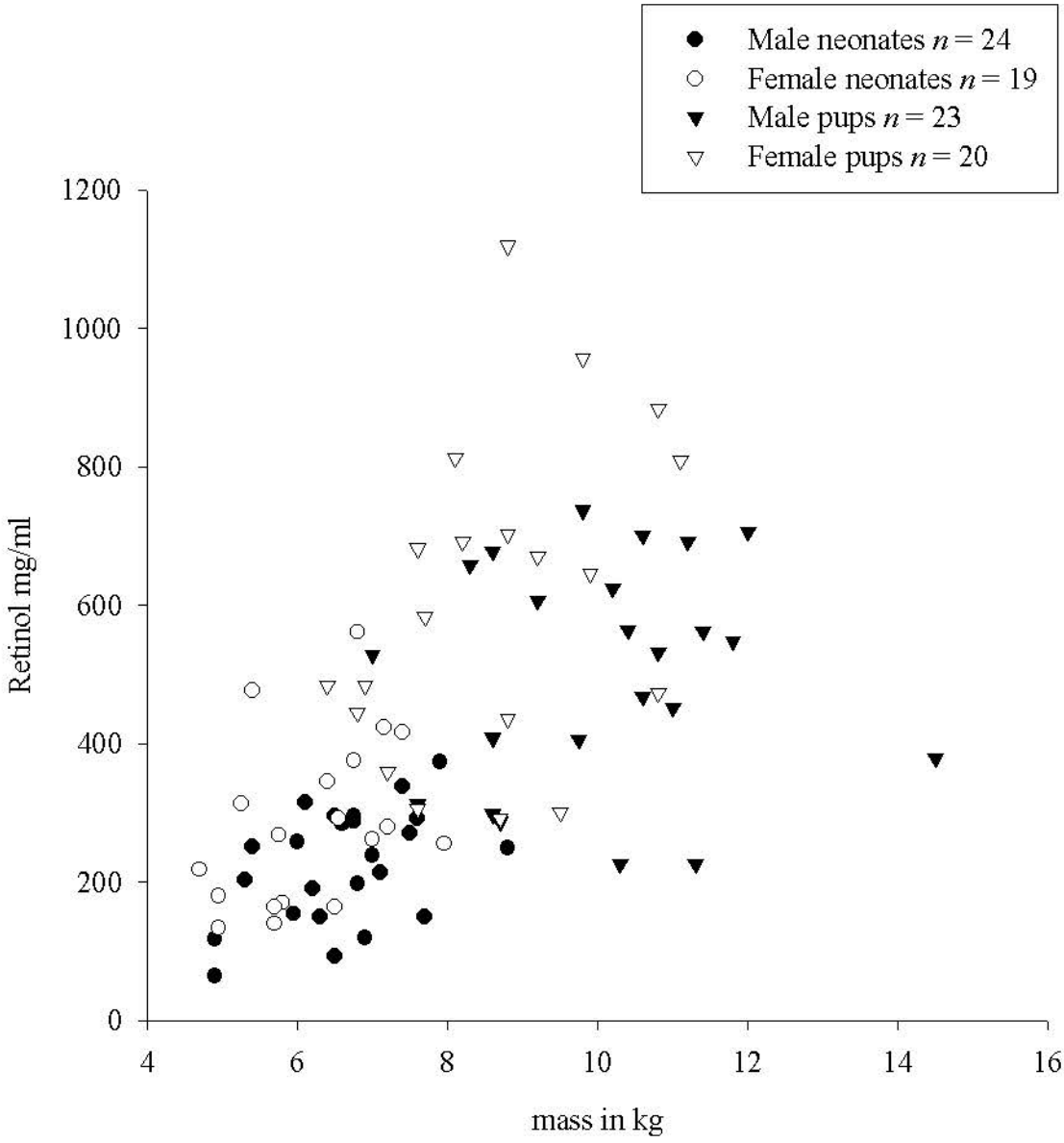

Figure 4.1. Retinol levels in the serum of northern fur seal neonates and as pups 29 to 51 days later on St. George Island, Alaska, 1996.

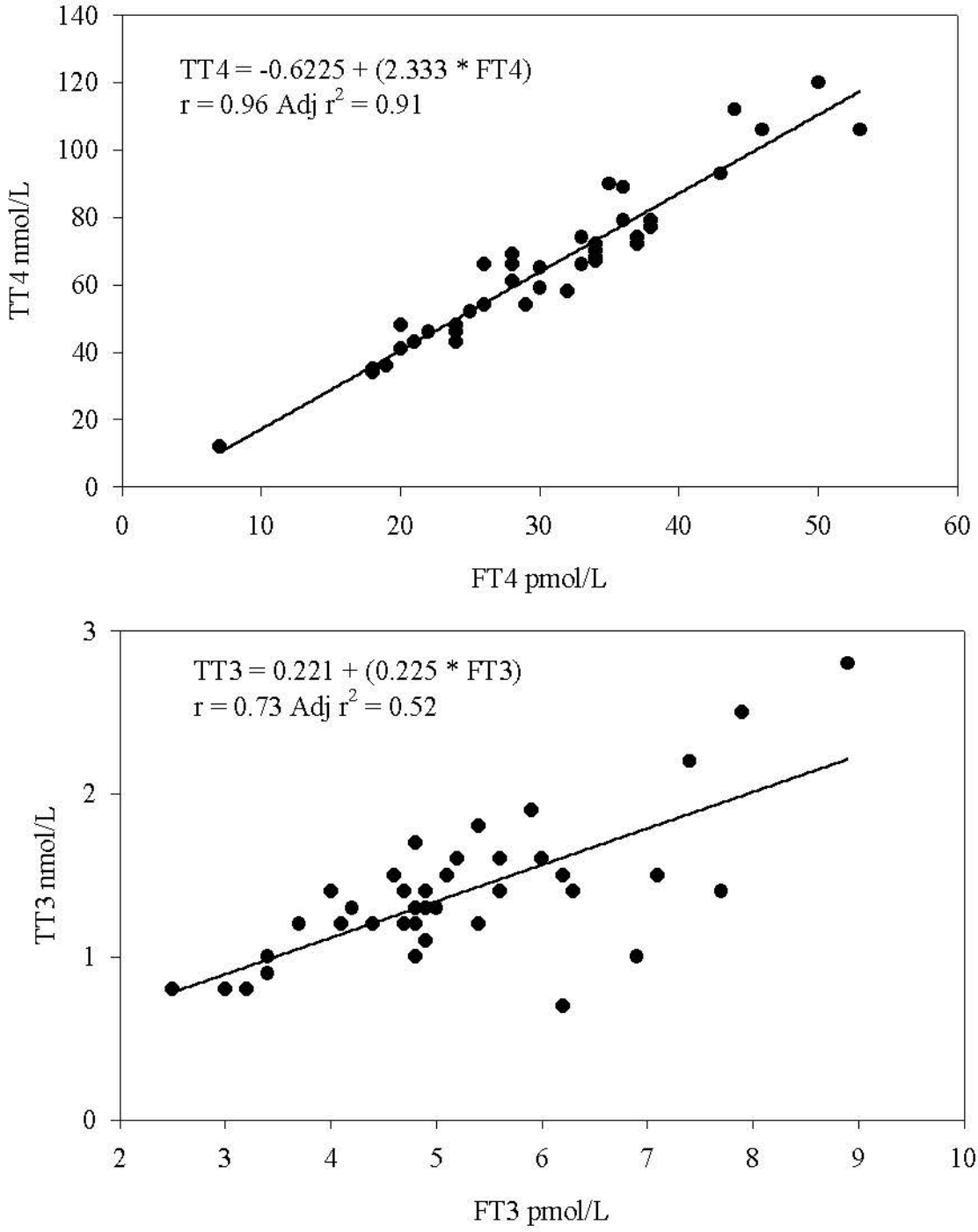

Figure 4.2. Regression equations of serum free thyroid hormones and bound thyroid hormones (T4 upper plot, T3 lower plot) in 42 northern fur seal pups on St. George Island, Alaska, 1996.

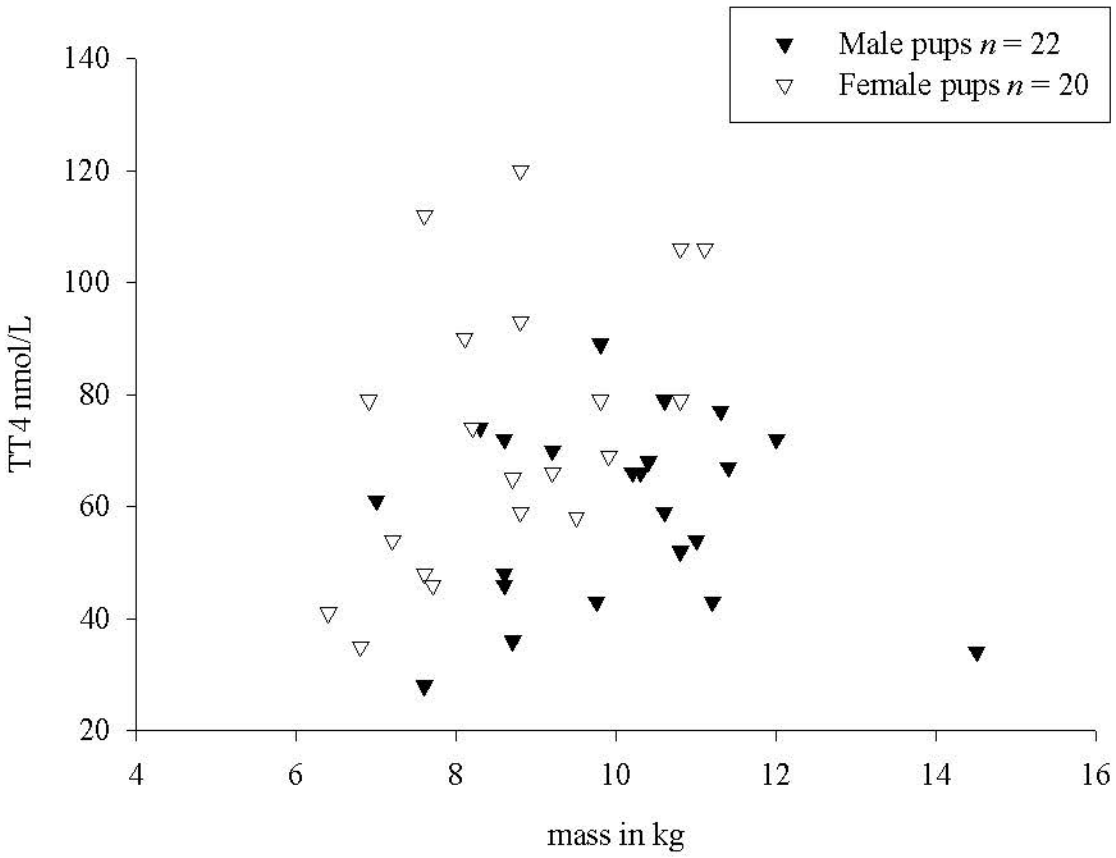

Figure 4.3. Total thyroxine (TT4) in serum versus body mass of northern fur seals pups on St. George Island, Alaska, 1996.

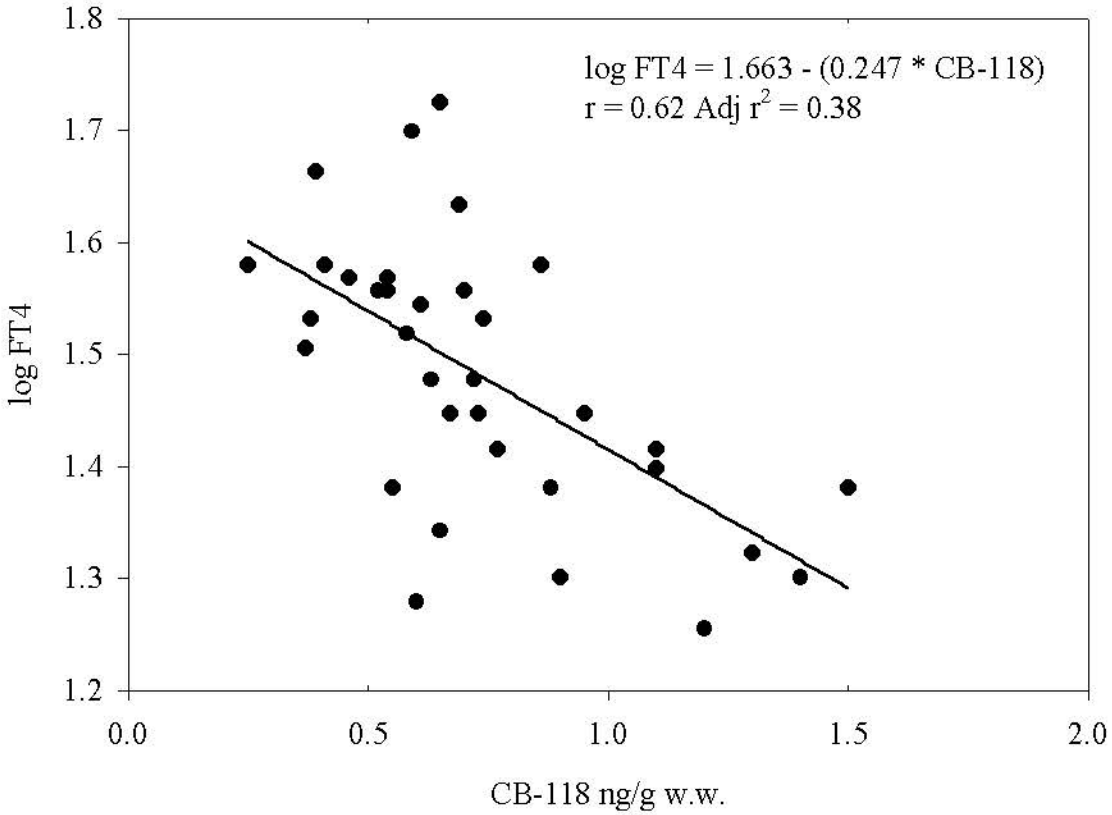

Figure 4.4. Regression equation of serum FT4 and blood concentration of CB-118 (w.w.) in 34 northern fur seal pups on St. George Island, Alaska, 1996.

Chapter 5

Effects of Parity and Dam Mass on Pup Mass, Length, Growth, and Condition in a Cohort of Northern Fur Seal Pups (*Callorhinus ursinus*)

5.0 Introduction

Breeding rookeries for more than 72% of the world's population of northern fur seals (*Callorhinus ursinus*) are located in the Bering Sea on the two largest Pribilof Islands, St. Paul and St. George, Alaska (Loughlin et al., 1994). During mid-March, pregnant fur seals begin a migration back to the Pribilof Islands from foraging in the North Pacific, arriving on the rookeries approximately three to four weeks after the arrival of territorial males in May (Bigg, 1990). The oldest females arrive first followed by successively younger females. Parturition occurs within 1 - 2 days after arrival on the rookery during late June to early August (Gentry, 1998). A brief estrus occurs approximately 6 days postpartum but embryonic diapause (delayed implantation) lasts until the end of the four months of lactation. Within a day following estrus, foraging trips begin (2 - 7 days in duration) and alternate with 1-3 day nursing bouts until the pup spontaneously weans between late October to November and then migrates (Gentry and Holt, 1986; Gentry, 1998). During the winter, pregnant fur seals and juveniles of both sexes feed throughout the North Pacific including continental shelf areas off North America, Russia and Japan (Bigg, 1990).

The current Pribilof stock abundance is less than half of historical levels and is listed as depleted under the Marine Mammal Protection Act (Loughlin et al., 1994; York

et al., 1997). The St. George subpopulation underwent an unexplained decline of 4 - 6% per year for more than a decade up to 1996 (York et al., 1997). Long-term monitoring of population trends suggest that the decline was due, at least in part, to increased post-weaning mortality at sea (Trites and Larkin, 1989; Trites, 1992).

Northern fur seal females sexually mature and give birth to their first pup at about age five (York, 1983). For the first two years of lactation, foraging trips of young dams are longer and more variable than those of older, multiparous dams (Gentry, 1998). In 1985, it was determined that survival to weaning was lower in pups born to young dams (Goebel, 1988). This study investigates whether a cohort of pups born to young, primiparous dams are born smaller and grow slower to 2 months-of-age than pups born to older, multiparous dams.

5.1 Materials and Methods

Study site

Northern fur seals were captured on four of the six rookery beaches of St. George Island, Alaska (N56° 34’ W169° 41’), one of the two main rookery islands in the Pribilof Island group in the Bering Sea. The treeless, wind-swept sub-arctic island is located 31 km from the continental shelf break. The rookery beaches are composed of sand, gravel and boulders of volcanic origin. During the fur seal occupation from May to November, the weather is typically overcast and marked by rain and fog with mean air and water temperatures around 5°C.

Animal criteria, capture, and marking techniques

Northern fur seal neonates (n = 49) and dams (n = 21) were captured between 13 July – 3 August 1996 with a noose pole extended from a 3-person roving 'blind box' constructed of plywood (approximately 1 x 1.5 x 2m) using methods detailed elsewhere (Antonelis, 1992; Boltnev et al., 1998). This 'blind box' afforded protection of personnel from aggressive territorial bulls and allowed access to most of the rookery with minimal animal disturbance.

All neonates were accompanied by their dams at the time of initial capture and dams were classified as young (≤5 years of age and likely to be primiparous) or old (>7 years, assumed to be multiparous) by body size, pelage and vibrissae characteristics as described by Vladimirov and Nikulin (1993). Neonatal status (estimated age ≤ 7 days) was based on the condition of the umbilical cord remnant or the degree of umbilical healing, neonatal behavior and observation of characteristic periparturient/pre-estrus behavior of the dam (Gentry, 1998). Although the neonates were observed in contact with their dams when selected for capture, the timing, duration and volume of their most recent suckling bout was not known.

Prior to release, each of the neonates was marked with a unique symbol by clipping the guard hairs on the top of the head and applying gel hair bleach (Clairol Beyond Blond®). A cohort of 42 pups was recaptured between 24 August and 12 September 1996. Pups were recaptured either as individuals using stealth and a net or by herding all pups on a section of the rookery.

At the time of capture, the mass of each pup was determined by placing the pup in a burlap sac or bucket and suspending it from a spring scale. The weight, minus the tare weight, was recorded to the nearest 0.2 kg. Standard length was determined by restraining the pup in ventral recumbancy and measuring to the nearest 0.5 cm from the tip of the nose to the tip of the tail. Girth was measured under the foreflippers at the level of the axilla and recorded to the nearest 0.2 cm during exhalation. Dams were restrained and weighed while suspended from an electronic scale as per standardized fur seal research techniques and the mass recorded to the nearest 0.2 kg (Antonelis, 1992).

A body condition index (CI) was calculated using the methods of Trites and Bigg (1992). The relationships by sex between length and mass for pups weighed during the perinatal period and at the end of the early developmental period were used to predict the mass from their measured lengths. Each of the four regression equations was used to calculate the expected mass and the ratio of observed to expected mass is the condition index. Pups that weigh more than expected have a condition index > 1.0, those in poorer condition, have a CI of < 1.0.

At initial capture (perinatal period):

CI for males = mass / (-10.89 + 0.252 * length)

CI for females = mass / (-6.436 + 0.182 * length)

At recapture (early developmental period):

CI for males = mass / (-21.128 + 0.383 * length)

CI for females = mass / (-13.069 + 0.275 * length).

Statistics

Descriptive statistics were calculated for mass, length and girth at perinatal capture and recapture. Pup masses were plotted against dam mass at the perinatal capture. Growth rates were determined by changes in size and days between captures. Results were grouped and compared according to pup sex, rookery, relative dam age, perinatal capture or recapture and days between capture. Student's *t*-tests and analysis of variance were used to detect differences between groups. Correlation coefficients were calculated by a Pearson Product Moment Correlation. Statistical analyses were performed using Sigma Stat 2.0® and SPSS 8.0® (SPSS, Chicago, IL). Results are reported as mean and standard deviation (SD), or standard error of the mean (SE), and the significance level was set at $p < 0.05$.

5.2 Results

The cohort initially captured consisted of 27 males and 22 females. Paired data were collected from dams (10 young, 11 old) captured along with their pups. Two female pups at initial capture had healed umbilical scars and were judged by dam/pup appearance and behavior to be suckling in the attendance period after the first foraging trip. These two were still likely to be less than two weeks of age and were included in the perinatal cohort data except where designated. Of the neonates initially captured, 22 males and 20 females were recaptured as pups 29 to 51 days later.

Male neonates were 7% heavier and 2% longer than females during the perinatal capture, but not significantly different in girth (Table 5.1). At recapture, all three

morphometric differences were significant with male pups 15% heavier, 3% longer and 6% larger in girth than female pups (Table 5.1). Male pups also gained more mass than females (mean 3.4 ± 1.2 versus 2.5 ± 0.8 kg, $p = 0.010$).

Young dams gave birth an average of 11 days later than old dams ($p < 0.001$). Estimated mean date of birth for pups of old dams was July 12 ($n = 25$) and July 23 for pups born to younger dams ($n = 24$). Old dams were heavier (mean 36.0 ± 5.0 kg) than young dams (mean 26.6 ± 2.8 kg, $p < 0.001$).

Figures 5.1 and 5.2 plot the weight of the dam against the weight of her pup during the perinatal period and at recapture in the early developmental period, respectively. The non-linear relationships suggest that heavier dams up to 35 kg have heavier pups at the end of the early developmental period compared to the lighter, and presumably younger, dams. However, for the largest and oldest dams (weighing more than 35 kg), there appears to be a decline in the weight of their pups. When male and female pups were compared by the age of there dam, there were not significant differences between the mass of pups born to young or old dams (Table 5.2). The only significant difference in measurements between pups born to young and old dams was a greater girth in female neonates (Table 5.2).

Growth rates (change in length and mass per day) were calculated between the perinatal period (1 - 7 days) and the end of the early developmental period (35 - 50 days). An average of 39 ± 6 days (range 29 - 51 days) elapsed between the first and second weighing. Growth rates for pups born to older females were estimated over a longer period of time than for pups born to younger females (42 days versus 35, $p < 0.001$).

However, there was no apparent relationship between growth rates and elapsed time at this stage of pup development (Figs. 5.3 and 5.4).

Analysis of variance was used to determine if there was a difference between the rate of growth of pups by sex and by age of dam. The number of days between weighings was not included as a covariate since there was no apparent relationship between time and growth rate.

Male growth (kg/day) differed significantly from females (males: mean 0.086 kg/d; females: mean 0.068 kg/d, $p = 0.037$). However, there was no significant difference when growth was measured as a change in body length (males: mean 0.27 cm/d; females: mean 0.27 cm/d, $p = 0.591$) and growth rates of pups (Table 5.3) raised by old or young dams (mass, $p = 0.973$; length, $p = 0.102$).

The average growth in length (AGL) was negatively correlated with perinatal length for both males and females ($r = -0.52$ and -0.51 respectively, $p < 0.02$). No correlation between average growth in mass (AGM) and perinatal mass was detected.

The mean condition index for males and females was 1.0, and did not differ significantly between sexes in either time (perinatal period, $p = 0.993$; early developmental period, $p = 0.999$). Nor was there a significant change in the condition of the pups from one period to the next (paired t-test, $p = 0.772$). However, the average condition of pups of old dams was slightly higher than the condition of pups of young dams in each period (mean 1.022 versus 0.976 in the perinatal period, $p = 0.047$; mean 1.027 versus 0.973 in the developmental period, $p = 0.049$; one tailed tests).

The survival rate to 40 days of age of this cohort was 85% for male pups and 86% for females (not significant, Chi-square = 0.0320, 1df, $p = 0.858$). One male suffered a carpal fracture, which subsequently abscessed and the pup became too weak to nurse. The other dead pups were severely emaciated. Of the males that died, two were pups of old dams and two were pups of young dams. Of the females that died, two were pups of old and one of a young dam. Survival rate was 88% for pups of young dams and 84% for pups of old dams but this difference was not significant (Chi-square, $p = 1.0$). Survival rate to 40 days was not predicted by neonatal weight, length, girth, dam age and dam weight.

The majority of the pups ($n = 28$, evenly divided by sex) captured for this study were born on *Staraya artil* rookery*;* seven males and three females on *Zapadni* rookery; three of each sex on *North* rookery*;* and three males/two females on *South* rookery, which is adjacent to *Zapadni* rookery. No significant differences between rookeries were detected for any of the data investigated.

5.3 Discussion

Sexual dimorphism at birth is expected and has been demonstrated in the highly polygynous northern fur seal (Costa and Gentry, 1986). Pups in this study show increasing dimorphism from initial capture in the perinatal period to recapture 4-7 weeks later at the end of the early developmental period.

In 1996, approximately 27,285 pups were born on St. George Island (York et al., 1997). Males (mean 9.99 kg, $n = 418$) during August 24 to 29 were significantly heavier than females (mean 8.33 kg, $n = 331$) (Towell et al., 1997). Mean lengths for the same

period were 81.9 cm for males and 78.0 cm for females. The pup lengths in this study are within these ranges but the females in the study cohort were slightly (mean 8.6 kg) larger than the mean for all females weighed on St. George.

The nonlinear relationships between neonate/pup weights and dam weights are consistent with the nonlinear relationship that occurs between dam age and fetus size in northern fur seals (Trites, 1991). Northern fur seals produce increasingly larger fetuses up to about 10 or 11 years of age (Trites, 1991). As the dams grow larger (over 35 kg) and older (> 11 years), they produce smaller pups. The three oldest dams (greater than 40 kg) shown in Figs. 5.1 and 5.2 are about 15+ years old based on growth curves in Trites and Bigg (1996). Particularly intriguing about these three individuals is that their increased foraging and maternal experience did not enhance the growth of their pups (as measured during the second weighing, Fig. 5.2). It is possible the mammary tissue of older dams is no longer able to produce the same quality or quantity of milk as younger dams.

The data show a significant sex difference in the growth rate of pups with males growing faster than females. There is no difference in growth rate between male or female pups born to young versus old dams. Boltnev et al. (1998) found significant differences between the growth rates of male and female pups. They estimated that northern fur seal pups on the Commander Islands grew at a rate of 0.08369 kg/d and 0.07084 kg/d (*sic*), for males and females, respectively, during the early development period (ages 11 - 40 d). These rates do not differ significantly from those found in this study (males, $p = 0.672$; females, $p = 0.656$). However, mean increases in body lengths

(AGL) calculated by Boltnev et al. (1998) (males: 0.374 cm/d; females: 0.343 cm/d) were significantly higher ($p < 0.001$).

Standard length is more difficult to measure accurately than mass. There is undoubtedly greater error in the estimates of body lengths taken from struggling pups, than body mass. Also, pups in this study were measured in a restraining device using a tape instead of being stretched on a marked board. The difficulty in obtaining an accurate length measurement along with fewer time points and relatively smaller sample size, may explain the lack of a significant difference between males and females within this study or the differences to the Boltnev et al. (1998) study.

Pups of old dams had a slightly higher body condition index than pups of young dams but survival rate during the observation period was not different. The lack of a significant difference in survival rate was contrary to expectations from survival rates determined in a 1985 cohort by Goebel (1988). During a study of foraging behavior and reproductive success on St. Paul Island, Goebel found that of 26 young dams, 9 had lost their pups by October and 78% lost their pups in the first 3 weeks after parturition while only 2 of 27 old dams lost their pups by October. Ultimate survival rates to weaning and after post-weaning migration for the 1996 cohort are unknown but all observed mortalities occurred within the first 3 weeks of life.

Low birth mass has been associated with increased pre-weaning mortality and lower return rates after the initial natal migration (Calambokidis and Gentry, 1985; Baker and Fowler, 1992; Boltnev et al., 1998). In this study, perinatal weight or sex of the pup did not affect survival through the early development period. Boltnev et al. (1998) found

in the 8 years of following large numbers of tagged pups on Bering Island, Russia, that survival to 40 days ranged from 80 to 95% with no significant differences between males and females. The survivors were significantly heavier, longer and had a higher CI at birth than non-survivors. The investigators also determined that in years with moderate or higher survival, pups born later in the birthing season had a significantly higher survival rate. In the 1996 cohort from this study, pups of young dams were born 11 days later than pups of old dams but there was not a statistically significant difference in survival rate albeit the sample size was small in comparison.

Being a young, presumably primiparous dam, did not significantly affect pup size or growth in this cohort over the observation period in this study. Sex of the pup was significantly correlated with size, growth and condition, but not survival. When pups born to dams of any age are considered together, this cohort did not vary greatly from other pups born on St. George Island in 1996 nor from those of previous studies of northern fur seal cohorts.

Table 5.1. Mean ± SD of morphometric measurements of northern fur seal pups as neonates and 29 - 51 days later as pups, on St. George Island, Alaska, 1996.

	Male neonates	Female neonates	Male pups	Female pups
Sample size	n = 27	n = 22	n = 22	n = 20
Mass (kg)	6.5 ± 0.8*	6.1 ± 0.9	9.9 ± 1.4†	8.6 ± 1.4
Length (cm)	70.6 ± 3.2*	69.0 ± 3.5	81.2 ± 3.0†	79.1 ± 3.6
Axillary girth (cm)	43.1 ± 3.6	42.8 ± 3.1	52.5 ± 3.6†	49.7 ± 3.8

*indicates significantly greater than female neonates, $p < 0.05$

†indicates significantly greater than recaptured females

Table 5.2. Mean ± SD of morphometric measurements of northern fur seal pups as neonates and 29 - 51 days later as pups, by dam age on St. George Island, Alaska, 1996.

Measure	Dam age	Male neonates	*n*	Female neonates	*n*	Male pups	*n*	Female pups	*n*
Mass (kg)	young	6.3 ± 1.1	10	5.8 ± 1.0	14	9.3 ± 1.4	8	8.4 ± 1.6	13
	old	6.7 ± 0.6	17	6.5 ± 0.7	8	10.2 ± 1.4	14	9.1 ± 1.0	8
Length (cm)	young	69.7 ± 2.6	10	68.5 ± 3.9	14	80.9 ± 3.0	8	78.5 ± 3.6	13
	old	71.1 ± 3.3	17	69.8 ± 2.3	8	81.3 ± 3.2	14	80.1 ± 3.7	8
Girth	young	40.6 ± 4.1	10	41.3 ± 2.9	14	52.4 ± 3.4	8	49.5 ± 4.3	13
	old	44.5 ± 2.2	17	45.4 ± 2.1*	8	52.5 ± 3.8	14	50.2 ± 2.7	8

*indicates significantly greater than neonates of young dams, $p < 0.001$

Table 5.3. Mean ± SD growth rate in mass and length for males and females through the early developmental period of northern fur seal pups on St. George Island, Alaska, 1996.

Measure	Dam age	Male pups	*n*	Female pups	*n*
Mass (kg/d)	young	0.079 ± 0.028	8	0.072 ± 0.026	13
	old	0.090 ± 0.026	14	0.062 ± 0.021	7
Length (cm/d)	young	0.301 ± 0.086	8	0.280 ± 0.087	13
	old	0.256 ± 0.063	14	0.246 ± 0.079	7

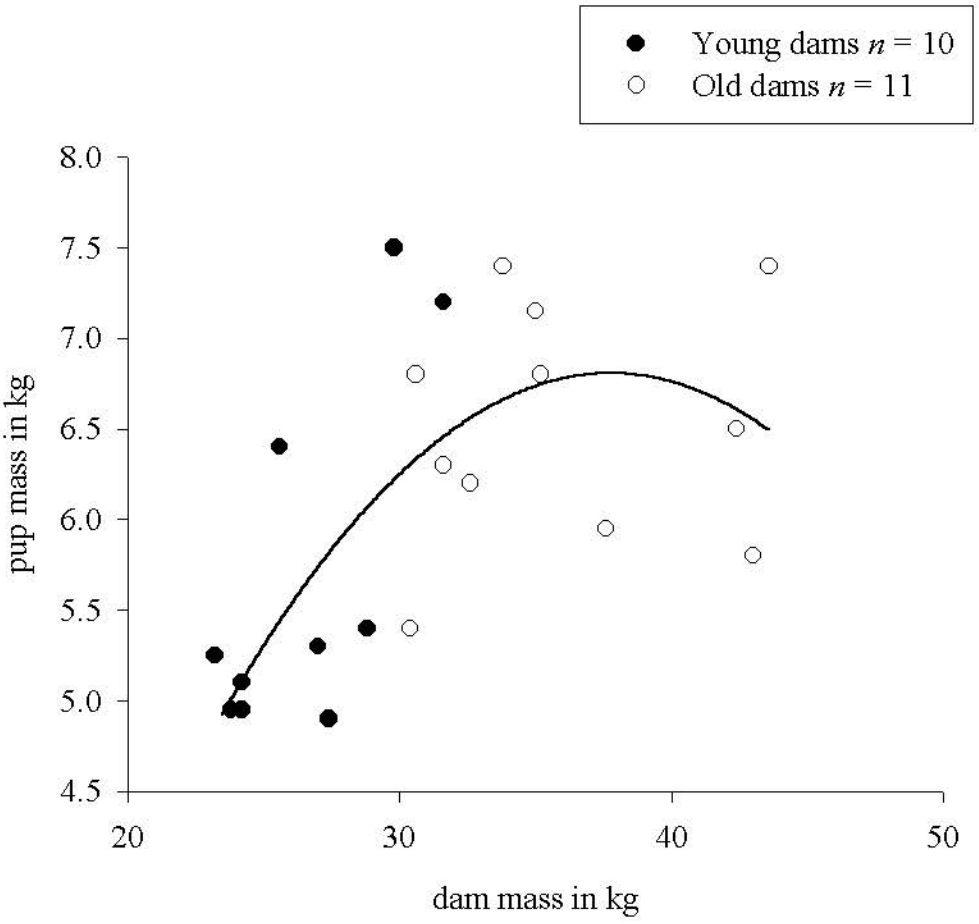

Fig 5.1. Neonatal pup mass versus dam mass of northern fur seals by age of dam from St. George Island, Alaska, 1996. The solid line represents the quadratic relationship, $p = 0.004$, $r^2 = 0.47$. Old dams are significantly heavier than young dams.

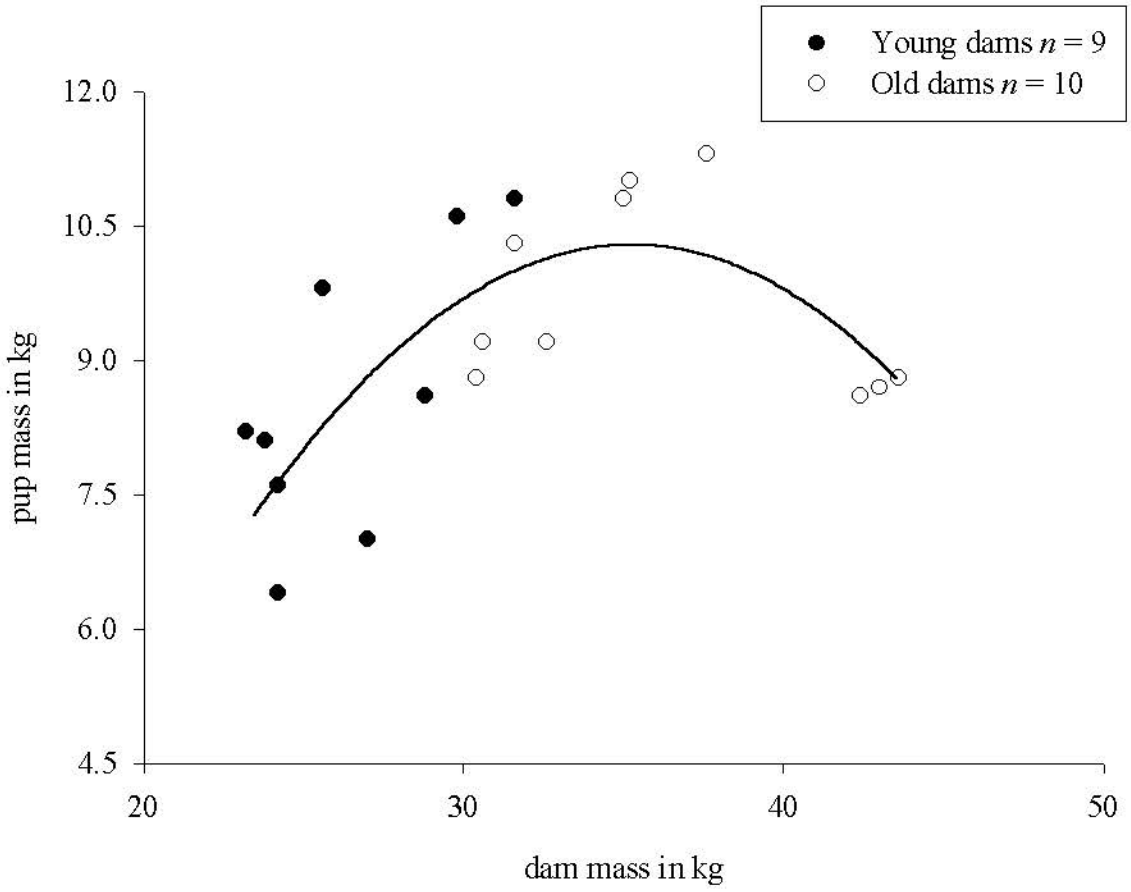

Fig 5.2. Pup mass at recapture versus dam mass at perinatal capture of northern fur seals separated by age of dam from St. George Island, AK, 1996.
The solid line represents the quadratic relationship, $p < 0.002$, $r^2 = 0.55$.

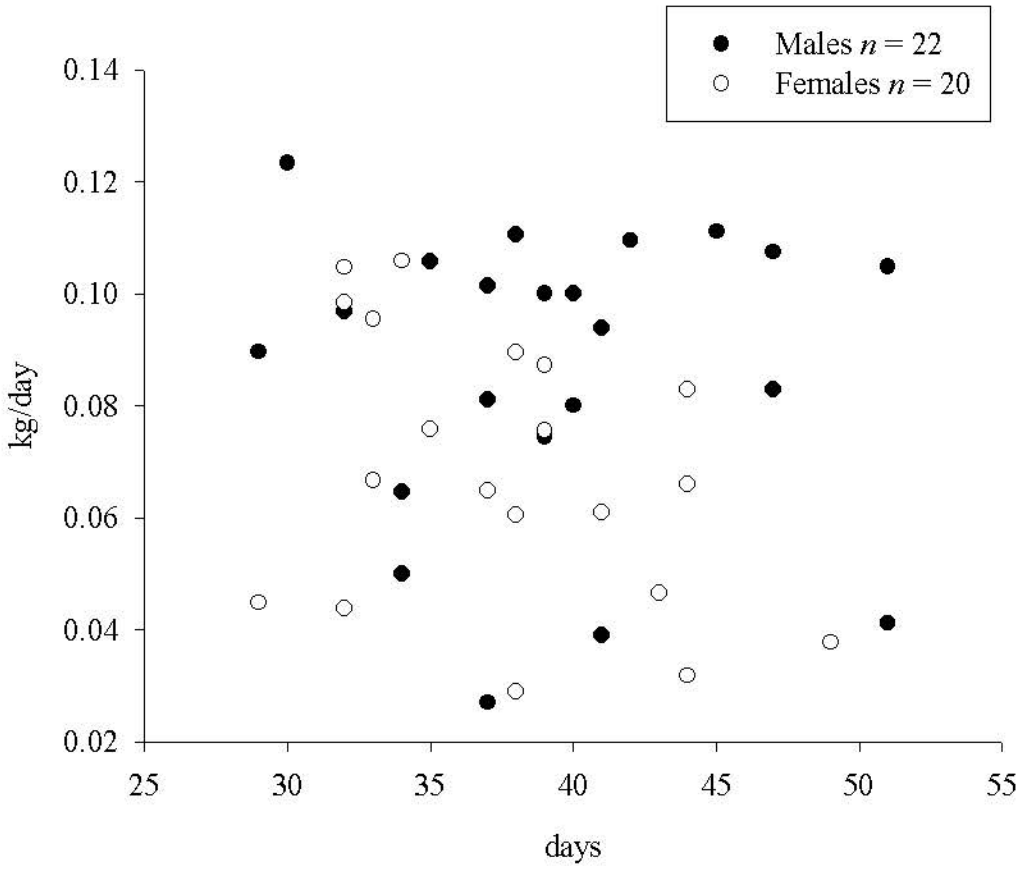

Fig 5.3. Growth rate (kg/day) versus the number of days from perinatal weighing to re-weighing 29 - 51 days later for 42 northern fur seal pups on St. George Island, 1996.

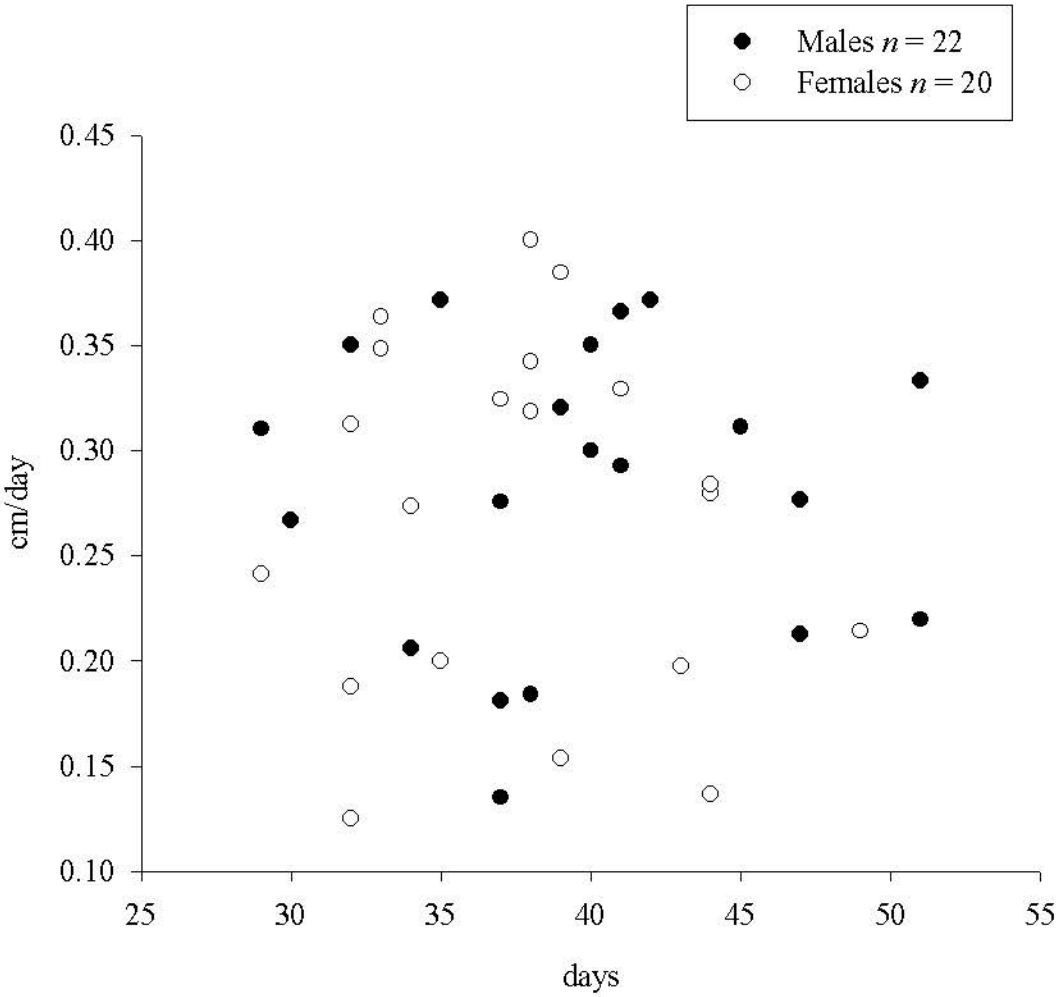

Fig 5.4. Growth rate (cm/day) versus the number of days from perinatal weighing to re-weighing 29 - 51 days later for 42 northern fur seal pups on St. George Island, 1996.

Conclusion

This study used multiple assays concurrently in an assessment of the health and immune function and organochlorine (OC) exposure levels from milk to blood in free-ranging northern fur seal pups. This study used small amounts of whole blood instead of depot tissues (i.e. blubber) obtained by more invasive procedures for the determination of OC concentrations. The cryopreservation of lymphocytes in a remote field setting was demonstrated to be feasible, allowing a cellular immune function assessment that previously could only be done on animals located in convenient proximity to the immunology laboratory.

Neonatal OC contaminant exposure through milk ingestion was greater for pups born to primiparous dams than pups born to multiparous dams. The differential milk excretion of accumulated OCs by parity has been widely speculated but rarely demonstrated. The first-born pups have higher levels of OCs in their peripheral blood up to at least 2 months into the nursing period, but the highest levels are found during the perinatal period. Significant "bioaccumulation" of multiple PCB congeners had already taken place in the pups within days of birth.

The number of samples available for lymphoproliferative immunoassays were limited and responsiveness to mitogen stimulation was lower in neonates with higher blood OC levels. In lymphocyte samples from older pups, a correlation with exposure levels could not be examined because all samples available for testing had poor proliferative responses. Lack of prior baseline or captive studies to document normal lymphoproliferative responses under controlled conditions, severely limited the

interpretation of the assays performed with this cohort but future studies utilizing cellular immune function analyses are warranted and feasible.

As part of the overall health assessment, traditional indices such as total white blood cell counts, differential cell counts and haptoglobin levels showed few differences. These types of indices are non-specific measures of inflammation and not typically affected by OC exposure in lab animals so the present findings are consistent with other studies. These parameters are measured to help detect other underlying disease processes that may affect the interpretation of the contaminants and immune function data. Body condition, mass, growth rates and survival rates varied only slightly by the age of the dam and most significant differences observed were a function of the pup's gender. Gender seemed to have very little affect on any of the other indices examined.

Serum retinol and thyroxine levels are typically reduced in concentration in OC toxicosis in mammals. Serum retinol and thyroxine levels were negatively correlated to OC concentrations in blood of pups and milk of their dams. The total immunoglobulin levels measured during the perinatal period in this study are contrary to a previous study. The Ig levels of the 1996 cohort indicated there is generally high maternal transfer, at least in pups born to old dams. No pre-colostral serum samples were available so transplacental vs. colostral transfer could not be assessed. First-born pups apparently received less maternal antibody than subsequent pups. Using a more specific *in vivo* assessment of humoral immunity, antibody response to tetanus vaccination, was more useful than total Ig levels. There were large differences in the proportion of pups

responding to the vaccine with pups of old dams having low OC exposure and better antibody responses.

The findings indicate that first-born fur seal pups have higher OC exposure, poorer immune responses, and thus might be at higher risk of morbidity and mortality from pathogens encountered before and after weaning than pups born to multiparous dams. Survival to the end of the early developmental period was apparently unaffected by OC exposure. Evidence of substantial OC contaminant exposure at a critical period of development for the immune system and evidence of adverse affects on the immune system must be considered as contributing factors to the population decline and lack of recovery of northern fur seals breeding on St. George Island.

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Appendix A. PCB structure and abbreviations (Ballschmiter and Zell, 1980)

IUPAC No.[a]	Chlorine substitutions	No. of *ortho* chlorines	TEF[b]
	Tetrachlorobiphenyls		
77	3,3,',4,4'	0	0.0005
	Pentachlorobiphenyls		
101	2,2',4,5,5'	2	na[c]
105	2,3,3',4,4'	1	0.0001
118	2,3',4,4',5	1	0.0001
126	3,3,4,4',5	1	0.1
	Hexchlorobiphenyls		
128	2,2',3,3',4,4'	2	na
138	2,2',3,4,4',5'	2	na
153	2,2',4,4',5,5'	2	na
156	2,3,3',4,4',5	1	0.0005
157	2,3,3',4,4',5'	1	0.0005
169	3,3',4,4',5,5'	0	0.01
	Heptachlorobiphenyls		
170	2,2',3,3',4,4',5	2	0.0001
180	2,2',3,4,4',5,5'	2	0.00001
189	2,3,3',4,4',5,5'	1	0.0001

[a]International Union of Pure and Applied Chemistry

[b]TCDD Toxic Equivalency Factor

[c]na indicates not assigned a TEF by World Health Organization-European Centre for Environment and Health