IMMUNOLOGICAL AND HEMATOLOGICAL BIOMARKERS FOR CONTAMINANTS IN FISH-EATING BIRDS OF THE GREAT LAKES

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(ABSTRACT)

Field and laboratory investigations have demonstrated that halogenated aromatic hydrocarbons (HAHs), which include PCBs and dioxin, are associated with developmental and population-level problems in fish-eating birds of the Great Lakes. Other studies have shown that perinatal exposure to HAHs causes thymic atrophy and suppresses T lymphocyte function in laboratory animals. Higher exposure suppresses antibody production and alters white blood cell (WBC) counts. This study investigated whether persistent contaminants alter immunocompetence in Great Lakes herring gulls (*Larus argentatus*) and Caspian terns (*Sterna caspia*). It also evaluated the use of various immunological tests as biomarkers for contaminant-associated health effects in wild birds. Masses of immune organs and WBC counts were assessed in herring gull chicks at 11 colonies and adults at 13 colonies, including two colonies outside the Great Lakes. T-cell- and antibody-mediated immune functions were assessed in chicks at five sites for each species. This ecoepidemiological study revealed a strong association between persistent contaminants and suppression of T-cell-mediated immunity. In herring gull chicks, thymus mass decreased as the activity of liver ethoxyresorufin-O-deethylase (EROD), an index of HAH-exposure and Ah-receptor activation, increased. In Caspian tern and herring gull chicks, the phytohemagglutinin skin test for T cell function showed a strong negative exposure-response relationship with organochlorines. There was no discernible association between contaminants and suppression of antibody-mediated immunity as

measured by the sheep red blood cell antibody test and bursal mass. However, contaminant effects on bursal mass were confounded by fluke infections. Several WBC variables in both species were associated with contaminants, but the evidence was weaker than for effects on T-cell-mediated immunity. The identity of the particular organochlorine(s) responsible for alterations of T cell function and WBC counts could not be determined because concentrations of organochlorines were highly co-correlated in bird tissues. However, PCBs were the most likely cause because of their high concentrations and immunotoxic potential. Tests of immune function, WBC counts, and immune organ masses are useful biomarkers for assessing health effects, including those associated with contaminants, in wild birds.

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mine. I will always cherish the many memories from our time spent on the lakes--from sharing beautiful sunsets on calm evenings to being marooned on remote islands by stormy seas--and I look forward to making many more memories.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Ah	aryl hydrocarbon
AHH	aryl hydrocarbon hydroxylase
ANCOVA	analysis of covariance
ANOVA	analysis of variance
B lymphocyte	bursa-derived lymphocyte
CATE	Caspian tern
C-TEQs	TCDD toxic equivalents determined by the chicken hepatocyte bioassay
CYP1A1	cytochrome P450 mono-oxygenase 1A1
DDD	1,1-dichloro-2,2-bis(*p*-chlorophenyl)-ethane
DDE	1,1-dichloro-2,2-bis(*p*-chlorophenyl-ethylene
DDT	1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-ethane
EDTA	ethylenediaminetetraacetic acid
EROD	ethoxyresorufin-O-deethylase
GC/MSD	gas chromatograph/mass spectrometer detector
GLEMEDS	Great Lakes embryo mortality, edema, and deformities syndrome
HAHs	halogenated aromatic hydrocarbons
HCB	hexachlorobenzene
HEGU	herring gull
HG-TEQs	TCDD toxic equivalents calculated from herring gull-specific toxicity equivalency factors
H/L ratio	heterophil/lymphocyte ratio
HPLC	high-performance liquid chromatography
IgG	immunoglobulin G
IgM	immunoglobulin M
IUPAC	International Union of Physicists and Chemists
ODS	octadecylsilane
PBS	phosphate buffered saline
PCBs	polychlorinated biphenyls
PCDDs	polychlorinated dibenzo-dioxins
PCDFs	polychlorinated dibenzo-furans
PCV	packed (red blood) cell volume
PHA	phytohemagglutinin
SI	stimulation index (for PHA skin test)
SRBC	sheep red blood cells
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCDF	2,3,7,8-tetrachlorodibenzo-*p*-furan
TEF	toxicity equivalency factor
T lymphocyte	thymus-derived lymphocyte
WBC	white blood cell

Chapter 1. Problem Statement and Justification

Introduction

Despite attempts to control pollution over the past several decades, a variety of environmental contaminants continue to impact the Great Lakes of North America. In 1987 the Great Lakes Water Quality Board identified 11 critical pollutants that still present risks to human health and the aquatic ecosystem: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,7,8-tetrachlorodibenzo-*p*-furan (TCDF), benzo[a]pyrene (b[a]p), 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-ethane (DDT), dieldrin, hexachlorobenzene (HCB), alkylated lead, mirex, mercury, polychlorinated biphenyls (PCBs), and toxaphene (Colborn *et al.* 1990). Numerous studies have suggested that at least some of these contaminants present significant risks to a number of wildlife species, especially those that occupy high positions in the aquatic food web. Biomagnification of persistent, lipophilic chemicals produces high concentrations in the tissues of Great Lakes fish, reptiles, birds, and mammals, including humans, that eat contaminated fish. These contaminants cause abnormalities that are evident at biochemical, physiological, organismal, and population levels.

Environmental contaminants have been linked to deformities, mortality, and population effects in Great Lakes fish-eating birds, including gulls, terns, cormorants, herons, and eagles. Numerous studies have demonstrated that halogenated aromatic hydrocarbons (HAHs), which include PCBs, TCDD, and TCDF, are responsible for many of these bioeffects. Laboratory studies have shown that one of the most characteristic effects of exposure to HAHs and other pollutants found in the Great Lakes is immunosuppression. Increased susceptibility to infectious diseases potentially is an

important mechanism by which contaminants could produce mortality and population effects in fish-eating birds of the Great Lakes. This chapter reviews the past and present effects of contaminants on these birds as well as the laboratory evidence for HAH-induced immunosuppression. It then describes a research project that studied the effects of environmental contaminants on immune function in fish-eating birds of the Great Lakes.

Literature Review

During the mid 1960's, several studies suggested that environmental contaminants were disrupting reproduction in fish-eating birds in the Great Lakes ecosystem. Subsequent research confirmed these effects and contributed to restrictions on many of the more persistent chemicals. Although reproduction has recovered in many populations of piscivorous birds in this region, many effects on birds and other wildlife are occurring still, especially at highly contaminated sites. The literature documenting the biological effects of contaminants in Great Lakes wildlife is quite extensive, so only a cursory review will be given here. Other reviews on the subject can be found in Peakall *et al.* 1980; Mineau *et al.* 1984; Fox and Weseloh 1987; Peakall and Fox 1987; Gilbertson 1988, 1989; Peakall 1988; Colborn *et al.* 1990; Fox *et al.* 1991; Gilbertson *et al.* 1991; Government of Canada 1991; Fox 1993.

Effects of Environmental Contaminants on Fish-Eating Birds of the Great Lakes

Early Indications of Reproductive Problems

Reproductive failure and population declines in herring gulls (*Larus argentatus*) and double-crested cormorants (*Phalacrocorax auritus*) were the first indications that environmental contaminants might be affecting birds in the Great Lakes. Keith (1966) observed that adult herring gulls near Green Bay, WI, had organochlorine pesticide residues in fat that exceeded 2000 μg/g during 1963. During 1964, bird banders observed a lower number of herring gull chicks than during past years. Hatching success was only 41%, and eggshell damage was reported. Ludwig and Tomoff (1966) reported low reproduction in herring gulls in Traverse Bay, MI. Hatching success was only 28%, and 20% of the eggs were cracked. Both of these studies reported a fledging rate of only 0.3-0.4 fledglings/nest, compared to a normal rate of 1.2 fledglings/nest. Alterations in parental behavior also were observed.

These ecologists suggested that organochlorine contaminants might be causing these reproductive problems. Herring gull eggs from Green Bay contained twice as much DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl-ethylene; a toxic metabolite of DDT) than eggs from control sites (Keith 1966). Traverse Bay eggs did not differ from controls in DDE content, although Ludwig later suggested that PCBs might have confounded pesticide analysis. Unfortunately, TCDDs and TCDFs were not assayed (Gilbertson 1988).

Although populations of double-crested cormorants in the Great Lakes basin increased from the beginning of the century through the 1940's, declines were observed during the 1950's and 60's. Population size decreased 80% from peak numbers by 1972

(Peakall 1988). During the late 1960's and early 1970's, reproductive failure and 20-30% eggshell thinning were reported in double-crested cormorants from Lake Huron (Weseloh *et al.* 1983, Gilbertson 1988). DDE-induced eggshell thinning probably was the most important factor in double-crested cormorant population declines during this time period (Peakall 1988).

During 1972, five herring gull colonies in Lake Ontario produced only 0.06 to 0.21 fledglings/nest--approximately 1/10 of normal. DDE-induced eggshell thinning produced eggshell breakage and flaking in the most contaminated colonies (Gilbertson 1974). On Scotch Bonnet Island in Lake Ontario during 1973, 27% of herring gull nests had no eggs, 20% of eggs exhibited early embryonic mortality, hatching success was only 17%, chick mortality was 74%, and fledging success was only 0.06 fledglings/pair (Gilbertson and Hale 1974a,b). These problems were associated with high contaminant residues in the eggs: 140 µg/g (dry wt.) DDE and 550 µg/g PCBs. Dieldrin, heptachlor epoxide, and HCB also were detected.

Further Documentation of Problems

After these initial reports, many other studies documented associations between reproductive problems and high contaminant concentrations in piscivorous birds in the Great Lakes. Ingestion of HAHs by chickens (*Gallus domesticus*) causes a syndrome called chick edema disease, which includes subcutaneous, pericardial, and peritoneal edema as well as reduced body mass gain, hemorrhaging, liver necrosis, and even death (Gilbertson *et al.* 1991). Several outbreaks of similar symptoms were observed in fish-eating birds at highly contaminated sites in the Great Lakes during the 1960's and 70's. High embryonic mortality, subcutaneous, pericardial, and peritoneal edema, congenital

deformities, growth retardation, hepatomegaly, liver necrosis, and liver porphyria are the major symptoms associated with this condition in wild birds (Gilbertson 1989). Gilbertson *et al.* (1991) named this set of symptoms Great Lakes embryo mortality, edema, and deformities syndrome (GLEMEDS). The first occurrence of GLEMEDS was observed in Lake Ontario herring gulls between 1966 and 1976 (Gilbertson and Fox 1977, Gilbertson *et al.* 1991). Retrospective analysis of herring gull eggs from the lower Great Lakes showed that TCDD concentrations averaged 500 pg/g (parts per trillion) during 1974 but were as high as 1200 pg/g in earlier years (Gilbertson 1988). A second outbreak of GLEMEDS occurred in Forster's terns (*Sterna forsteri*) in Green Bay, WI, starting in about 1973 (Gilbertson 1989, Gilbertson *et al.* 1991, Kubiak *et al.* 1989).

Several important studies demonstrated that poor reproduction was caused not only by factors intrinsic to the egg (i.e., developmental toxicity of contaminants) but also by extrinsic factors (i.e. contaminant-induced abnormalities in parental behavior). Peakall *et al.* (1980) switched eggs between a contaminated herring gull colony on Lake Ontario and an uncontaminated colony in New Brunswick. During 1975, 86% of "clean" eggs incubated by "clean" adults hatched. "Dirty" eggs incubated by "clean" adults had low hatchability (only 10%), demonstrating intrinsic factors. But "clean" eggs incubated by "dirty" adults also had low hatchability (only 7%), demonstrating extrinsic effects. "Dirty" eggs incubated by "dirty" adults had a hatchability of only 2%. Fox *et al.* (1978) used telemetered eggs in Lake Ontario and New Brunswick to investigate the nature of these extrinsic effects. Lake Ontario herring gulls exhibited poor incubation behavior compared to New Brunswick controls. At the Lake Ontario colony, parents of unsuccessful nests left their nests unattended for three times longer than parents of successful nests. The average egg temperature was 1 °C less for unsuccessful nests compared to successful nests. The

length of time that a nest was left unattended was positively correlated with organochlorine concentrations in the egg.

Reproduction of bald eagles (*Haliaeetus leucocephalus*) also was affected by contaminants, primarily by DDT but possibly by PCBs also. Between 1968 and 1970, bald eagles on Lakes Superior, Michigan, Huron, and Erie averaged only 0.13 young/occupied nest. Production of 0.7 and 1.0 young/occupied nest are necessary for maintaining stable and healthy populations, respectively. On Lake Superior, 21% eggshell thinning was associated with egg concentrations of 57 μg/g (wet wt.) DDE and 28 μg/g PCBs (Postupalksy 1971).

Several studies indicated that organochlorine contaminants were affecting survival throughout the year as well as causing the decreases in reproduction noted above. Ludwig and Ludwig (1969) captured 15 nesting adult herring gulls from Traverse Bay, MI. Upon starvation, six gulls died within 10 days. In previous experiments, relatively uncontaminated herring gulls from Lake Huron withstood a 30% loss of body mass in 17 days without any mortality or loss of activity. Traverse Bay herring gulls that died during starvation had brain concentrations of 12 μg/g DDT, 6 μg/g DDD (1,1-dichloro-2,2-bis(*p*-chlorophenyl)-ethane; a metabolite of DDT), 180 μg/g DDE, and 2 μg/g dieldrin, suggesting the lethal mobilization of organochlorine pesticides from body fat during starvation. Sileo *et al.* (1977) analyzed organochlorine contaminants in the brains of ring-billed gulls (*Larus delawarensis*) from southern Ontario during 1969 and 1973. Ring-billed gulls found dying with signs of neurologic poisoning and ring-billed gulls found dead with no apparent cause of death had significantly higher brain organochlorine residues than healthy ring-billed gulls shot for comparison. Dieldrin, an organochlorine insecticide, was implicated as an important factor. Although they did not test the correlation

statistically, the authors noted an inverse relationship between brain organochlorine index and body mass, suggesting lethal mobilization of contaminants during post-nuptial or post-juvenile molt (most mortality occurred during late summer and early autumn). Alternatively, the inverse relationship could have been caused by organochlorine-induced anorexia and emaciation.

Restrictions on Organochlorines-- Improvements in Reproduction and Population Recoveries

During the 1970's and early 1980's, the use of most organochlorines was greatly restricted in the U.S. and Canada. In the U.S. DDT was banned in 1972. PCBs were banned in the U.S. in 1979, except for use in totally closed systems or by special exemptions. With reduced production of organochlorines, release of HCB into the environment decreased. Decreasing trends of organochlorine residues in wildlife through the late 1970's were associated with increased reproduction and population recovery in some species, including herring gulls and double-crested cormorants.

Mineau *et al.* (1984) reported significant decreases in DDT, DDE, and mirex concentrations in herring gull eggs from Lakes Ontario, Erie, Huron, and Superior. PCB and dieldrin concentrations decreased in herring gull eggs from Lakes Ontario, Erie, and Huron but not Superior. Weseloh *et al.* (1989) found that DDE and PCB concentrations in the eggs of common terns (*Sterna hirundo*) declined 80-90% in four colonies on Lakes Huron, Erie, and Ontario between 1969-73 and 1981.

In egg switching experiments during 1975, Peakall *et al.* (1980) had demonstrated severe intrinsic and extrinsic effects that were limiting reproduction in Lake Ontario herring gulls. During 1976, these researchers found only intrinsic effects. Neither intrinsic nor

extrinsic effects were observed during 1977. By 1978, the number of fledged herring gull young/breeding pair had increased to over 1.0 for Lakes Ontario and Erie, both of which had shown severe reproductive impairment during the early 1970's (Gilbertson 1988). By 1979-81 the Great Lakes double-crested cormorant population had begun to recover. Although 5-14% eggshell thinning was still observed, reproductive output had increased to a mean of 1.5-2.0 young/nest (Peakall 1988). In Lake Ontario, double-crested cormorant populations increased at an annual rate of 56% between 1974 and 1982 (Price and Weseloh 1986).

Continued Problems during the 1980's and 1990's

Despite the improvements observed during the late 1970's, a number of problems, especially at highly contaminated sites, have persisted to the present. Although concentrations of many contaminants declined drastically during the late 1970's, concentrations have declined slowly, leveled off, or increased during the 1980's and 90's (Government of Canada 1991). As biological and analytical chemical methods have improved, the research emphasis for Great Lakes toxics has shifted to looking at problems in finer detail. Much emphasis has been placed on the development and use of biomarkers, which are biochemical, physiological, or histological changes that measure effects of, or exposure to, toxic chemicals. Congener-specific chemical analysis has become an important tool in assessing the effects of complex mixtures of contaminants on birds, other wildlife, and humans. Although expensive, more detailed chemical analyses have helped to separate the effects of the 209 PCB congeners and 75 dioxin congeners, which vary widely in their toxicity and environmental persistence.

Many biomarkers have been proposed for use in monitoring exposure to, and effects of, persistent toxic chemicals in Great Lakes wildlife, but only a few will be discussed here. Cytochrome P-450 mono-oxygenases belong to a class of liver detoxification enzymes known as mixed function oxidases. When an animal is exposed to certain contaminants or drugs, the expression of certain cytochrome P-450's is induced. Hoffman *et al.* (1987) found that liver microsomal aryl hydroxylase activity (a specific cytochrome P-450 reaction) was three times greater in Forster's tern hatchlings from Green Bay, WI, as compared to those from Lake Poygan, a nearby reference site.

Some HAHs, including TCDD, TCDF, HCB, and PCBs, can inhibit heme synthesis and produce a buildup of highly carboxylated porphyrins in liver tissue. Fox *et al.* (1988) examined the concentrations of porphyrins in the livers of adult herring gulls collected throughout the Great Lakes during the early 1980's. Herring gulls also were collected from a control site in New Brunswick. The highest concentrations of porphyrins were found in herring gulls from the sites most heavily contaminated with HAHs (Green Bay, WI, Saginaw Bay, MI, and Lake Ontario). Liver porphyrins hold much promise as an early warning indicator of HAH toxicity since elevated porphyrin concentrations usually are observed before other toxic effects of HAHs in laboratory studies (Fox *et al.* 1988).

Retinol, or vitamin A, is essential for proper development, immune function, and vision. Environmental contaminants such as HAHs can disrupt vitamin A homeostasis (Zile 1992), and such effects have been observed in fish-eating birds of the Great Lakes (Spear *et al.* 1985, 1990; Fox 1993). Retinoids stored in the liver were lower for adult herring gulls from the Great Lakes as compared to those from the Atlantic coast. Although the greater amount of vitamin A in the marine food web contributed to this difference,

environmental contaminants also appeared to be important. Within the Great Lakes region, liver retinoid stores varied significantly among sites and between years.

Thyroid mass, histology, and hormones are other potential biomarkers for toxic effects of HAHs in Great Lakes wildlife (Moccia *et al.* 1986, Government of Canada 1991, Fox 1993). Adult herring gulls in the Great Lakes had larger thyroid glands than those from a less-contaminated colony on the Atlantic coast. In Lake Ontario, where gull tissues have been sampled over time, goiter has decreased at the same time that organochlorine contamination has decreased. At highly contaminated Great Lakes colonies, there was a high prevalence of epithelial hyperplasia, which was not observed at the reference colony. Concentrations of circulating thyroid hormones also might be affected by environmental contaminants.

Two recent studies have shown that environmental contaminants still are reducing reproduction in bald eagles in the Great Lakes region. Between 1983 and 1988, Kozie and Anderson (1991) found that bald eagles nesting on the shores of Lake Superior had a mean nest success of 57% and a mean of 0.8 young/occupied nest, compared to 77% nest success and 1.3 young/occupied nest for bald eagles nesting in inland Wisconsin. Reproduction of Lake Superior bald eagles had increased from the 10% nest success and 0.1 young/occupied nest during 1961-1970, but reproduction during 1983-1988 was still significantly lower for Lake Superior bald eagles compared to inland nesters. Contaminant concentrations, especially PCBs, were higher in dead nestling and immature bald eagles from Lake Superior compared to inland bald eagles of similar ages. Contaminant concentrations were relatively low in fish found in the diet of Lake Superior bald eagles. However, herring gulls, which comprised 48% of the bald eagles' diet, contained 16.95 µg/g (wet wt.) PCBs and 5.5 µg/g DDE. In another study, Bowerman *et al.* (1990) found

that blood plasma PCB concentrations were 6 times greater in bald eagle nestlings within 8 km of the Great Lakes as compared to nestlings further inland. Reproduction within 8 km of the Great Lakes was only 0.71 young/occupied nest as compared to 1.05 young/occupied nest further inland (Bowerman *et al.* 1990, Bowerman 1993). Pairs of nesting bald eagles on Lake Michigan and Lake Huron shorelines exhibit reproductive failure within 5 years of establishment, and a high turnover rate in the adult population is suspected (Government of Canada 1991). Bald eagle chicks with bill deformities also have been observed in the Great Lakes Basin: two during the 1960's, four during the 1980's, and three during 1993 (Bowerman 1993, Bowerman *et al.* 1994). A recent ecological risk assessment has concluded that PCBs and particularly TCDD-equivalents represent a significant reproductive risk to bald eagles living along the shorelines of the Great Lakes and rivers open to Great Lakes anadromous fish (Bowerman *et al.* 1995).

Reproductive problems, including GLEMEDS, have persisted for birds in Green Bay, WI. During 1983, congenital abnormalities, including crossed bills, were observed in herring gulls, common terns, double-crested cormorants, and Virginia rails (*Rallus limicola*) in Green Bay (Gilbertson 1989). During the same year, Kubiak *et al.* (1989) performed egg-switching experiments between Forster's terns colonies on Lower Green Bay and Lake Poygan, an inland control site. PCBs were the only contaminants present at concentrations high enough to cause the intrinsic (developmental effects) and extrinsic effects (parental inattentiveness) that were demonstrated by the egg-switching. "Dirty" eggs incubated by "dirty" adults had a hatching success that was 75% lower than that of "clean" eggs incubated by "clean" adults. This study was the first investigation to analyze PCB residues in tissues of Great Lakes birds on a congener-specific basis. Two PCB congeners, 2,3,3′,4,4′- and 3,3′,4,4′,5-pentachlorobiphenyl (PCB nos. 105 and 126),

appeared to occur at particularly high concentrations. The toxicity of these congeners, like other coplanar PCBs and TCDD, is mediated through the Ah receptor (Safe 1990). Kubiak *et al.* (1989) measured egg concentrations of specific PCB congeners, and then multiplied the concentration of each congener by a toxicity equivalency factor (a measure of toxicity relative to that of 2,3,7,8-TCDD, the most toxic compound that acts via the Ah receptor). They then calculated total TCDD-equivalence values based on the sum of the individual toxicities for each congener.

Another outbreak of GLEMEDS has been observed in double-crested cormorants and Caspian terns at contaminated sites in the upper Great Lakes, with particularly high frequencies of abnormalities in Upper Green Bay, Lake Michigan, and Saginaw Bay, Lake Huron (Gilbertson *et al.* 1991, Ecological Research Services 1991, Fox *et al.* 1991). Although this outbreak first was documented in 1986, it may have dated back to before 1967, which was the last year during which Caspian terns bred in Saginaw Bay until the early 1980's. Although double-crested cormorant populations have been exploding throughout much of the Great Lakes basin during the past decade, they have been experiencing extremely high frequencies of abnormalities in Upper Green Bay and did not successfully breed in Saginaw Bay until 1991. Fox *et al.* (1991) found that the frequency of bill defects in live double-crested cormorants was 52.1 abnormalities/10,000 chicks in Green Bay and 12.3/10,000 in the Beaver Islands (Lake Michigan) as compared to only 0.6/10,000 at reference colonies in the Canadian prairies. Dr. James P. Ludwig found an incidence of 1 bill defect/1000 Lake Michigan cormorant chicks, but found a frequency of 11.5/1000 eggs, leading to a total incidence of 12.5 bill defects/1000. Frequencies of other defects ranged from 2/1000 to 195/1000 in *dead* cormorant eggs (Gilbertson *et al.* 1991). Similar rates of defects were observed in Caspian terns. Such analyses are underestimates

of true rates of deformities because deformities cannot be detected in embryos that have died before 10 days of development and in partially decomposed eggs. The incidence of GLEMEDS in Caspian terns increased dramatically following a 100-year flood event during 1986 in Saginaw Bay. This flood mobilized highly contaminated sediments, causing an increase in PCB concentrations and reproductive and developmental problems (Ludwig *et al.* 1993).

Much research effort has been put into the development of *in vitro* bioassays to measure the TCDD-equivalent activity of environmental samples containing complex mixtures HAHs. One *in vitro* assay for TCDD-equivalents looks at the induction of specific cytochrome P-450 activity in rat hepatoma cells that are cultured in extracts containing HAHs (Tillitt *et al.* in press). The *in vitro* induction takes into account any synergistic, antagonistic, or additive effects that may occur in complex mixtures of HAHs. An alternative method that does not take into account interactive effects is to calculate total TCDD-equivalents by measuring concentrations of specific congeners and multiplying by toxicity equivalence factors. These toxicity equivalency factors are determined by comparing the LC_{50} or EC_{50} of each congener to that of TCDD in a variety of laboratory experiments (Safe 1990).

By using this *in vitro* induction assay for extracts of eggs from double-crested cormorants and Caspian terns, Tillitt *et al.* (1989, 1991) found that colonies with greater reproductive problems (Green Bay, WI, and Saginaw Bay, MI) had the highest concentrations of TCDD-equivalents. Other studies showed that the incidence of congenital abnormalities and other pathologies in double-crested cormorant embryos and chicks was strongly correlated with TCDD-equivalents (Ecological Research Services 1991; Giesy *et al.* 1994). During 1986-1988, Tillitt *et al.* (1992) collected eggs from 10 double-crested

cormorant colonies in the Great Lakes basin and from one control colony in Manitoba, Canada. They also monitored hatching success at these colonies. They used the rat hepatoma assay for TCDD-equivalents. They found a statistically significant negative correlation between total PCB concentrations and reproductive success ($r=-0.56$, $p=0.045$). However, the correlation between TCDD-equivalents and reproductive success was much stronger ($r=-0.84$, $p=0.003$). The authors noted that total PCB concentrations have declined 10 to 100 times from the mid 1960's, but now these concentrations have appeared to reach a steady state-- atmospheric inputs and internal cycling are balancing degradation. However, the PCB congeners that are found today in fish-eating birds are the more toxic forms with high TCDD-like activity. Indeed, Tillitt *et al.* (1992) found that the relative potencies of extracts from Great Lakes double-crested cormorant eggs were 3-4 times greater than the potencies of any PCB technical standards, which represent the mixtures originally released into the environment. This study provides the first clear documentation of relative enrichment of toxicity in PCB mixtures in Great Lakes wildlife. These more toxic congeners are more resistant to degradation and (or) are preferentially accumulated through the food web. Thus, although the negative impacts of DDT have decreased, PCBs are still a major concern for animals and people who eat fish from contaminated sites in the Great Lakes.

Although populations of herring gulls and Caspian terns have recovered on a basin-wide scale, the three species of Great Lakes terns (common, Caspian, Forster's) are exhibiting population-level effects in many areas. Long-term banding studies show that Caspian terns hatched on contaminated U.S. colonies (Green Bay and Saginaw Bay) return to breed at a rate less than half of that for terns hatched on cleaner Canadian colonies (Ludwig 1979, Mora *et al.* 1993). Indeed, the breeding population on these U.S. colonies

is supported by influx of young adult terns hatched on Canadian colonies. Similar population-level effects likely are occurring in other species, but there are less long-term banding data on which to test this hypothesis.

Summary

Extensive research spanning three decades has suggested numerous effects of environmental contaminants on fish-eating birds of the Great Lakes. During the 1960's and early 1970's, DDT and PCBs were the most important contaminants affecting Great Lakes wildlife on a regional scale. DDE-induced eggshell thinning was shown conclusively to be a causative factor in population declines of bald eagles and double-crested cormorants. The effects of PCBs and dioxins were less clear, although outbreaks of chick edema disease were probably caused by PCBs and (or) dioxins. Since the ban on many organochlorines, populations of some affected birds have recovered on the regional scale, but contaminants still cause problems, especially in heavily polluted areas. While the negative impacts of DDT have decreased, PCBs are still a cause for concern, especially since highly toxic PCB congeners with dioxin-like activity are accumulating in fish-eating birds. Continued monitoring using biomarkers of contaminant effects is important for assessing the effects of contaminants on the health of the Great Lakes ecosystem.

Immunosuppression Caused by Contaminants Found in the Great Lakes

Given this persistent problem of coplanar PCB congeners, it is important to realize that PCBs and other HAHs suppress normal immune function in laboratory birds and

mammals at concentrations comparable to those found in wild birds and mammals frequenting Great Lakes areas. Excellent reviews have been provided by Silkworth and Vecchi 1985, Thomas and Faith 1985, Luster *et al.* 1987, and Vos and Luster 1989. Effects often are more evident when developing embryos and young are exposed to contaminant concentrations lower than those needed to cause similar effects in adults (Thomas and Faith 1985, Vos *et al.* 1989). These effects on the developing immune system often persist long after exposure (Takagi *et al.* 1987, Holladay and Luster 1994). In addition to HAHs, other industrial chemicals as well as heavy metals and pesticides have immunotoxic effects in laboratory animals (Koller 1979, Caren 1981, Luster *et al.* 1987, Vos *et al.* 1989, Wong *et al.* 1992, Pruett 1994). Many of these immunosuppressive chemicals pollute the Great Lakes. The following discussion briefly reviews the immunotoxic effects of HAHs.

Various HAHs exert toxic effects on the immune system. In birds, the thymus and bursa of Fabricius are important sites for the maturation of T and B lymphocytes (types of white blood cells), respectively. The spleen is an important site for interactions between pathogens and lymphocytes. In laboratory animals exposed to lethal and sublethal doses of TCDD, the most consistent effect is thymic atrophy, which is characterized by a depletion of lymphocytes in the thymic cortex (Vos and Luster 1989). Similar effects have been observed in laboratory animals dosed with PCBs, especially mixtures containing a high percentage of coplanar congeners (Vos and Van Driel-Grootenhuis 1972, Silkworth and Grabstein 1982, Vos and Luster 1989). Harris *et al.* (1976) observed decreased spleen and bursa of Fabricius masses in the offspring of chicken hens fed PCBs. Although bursa mass was decreased, PCBs did not suppress the antibody response to *Brucella abortus*. Thymus masses were not reported. Coplanar PCBs and TCDD have produced dose-

dependent decreases in the number of viable lymphocytes in the thymus and bursa of Fabricius of chicken embryos that developed in eggs injected with coplanar HAHs (Nikolaidis *et al.* 1988a,b, Andersson *et al.* 1991). The number of lymphocytes was decreased in bursae that had been removed from a chicken embryo, exposed to TCDD or 3,3′,4,4′-tetrachloroazoxybenzene, and implanted into the chorioallantoic membrane of another embryo (Nikolaidis *et al.* 1990). Although the number of lymphocytes in the bursa was decreased, the bursal epithelium showed essentially normal development when examined microscopically. In chicken embryos, PCB congener #126 induces the activity of EROD in thymic and bursal tissue, demonstrating that these organs are targets for Ah-receptor-mediated toxicity (Lorr *et al.* 1992). At the same dose, EROD was approximately four times higher in the thymus than the bursa, suggesting that the thymus is more sensitive than the bursa to toxic effects mediated by the Ah receptor. At relatively high doses that produce other toxic effects, TCDD and PCBs can cause changes in the numbers of circulating lymphocytes (Vos and Van-Driel Grootenhuis 1972, Vos and Luster 1989). Tetrachlorodibenzo-*p*-dioxin also has caused changes in serum protein profiles, including decreased immunoglobulin concentrations (Loose *et al.* 1977, Sharma *et al.* 1978, Thomas and Hinsdill 1978, Hinsdill *et al.* 1980). It also has produced decreased hematocrit values, which indicate anemia (Hinsdill *et al.* 1980).

Humoral immunity involves the production of antibodies against foreign substances, including many pathogens. These antibodies help other components of the immune system to identify and destroy pathogens. The major effect of HAHs on humoral immune function is the suppression of the antibody response after acute exposure in adult animals. Tetrachlorodibenzo-*p*-dioxin and PCBs decrease antibody production and the number of antibody producing lymphocytes following challenge with an antigen (Sharma *et*

al. 1978; Thomas and Hinsdill 1978; Hinsdill *et al.* 1980; Clark *et al.* 1981, 1983; Silkworth and Grabstein 1982; Silkworth *et al.* 1984; House *et al.* 1990; Kerkvliet *et al.* 1990b). These HAHs appear to act directly on B lymphocytes, preventing their differentiation into antibody-producing plasma cells (Luster *et al.* 1988a).

Cell-mediated immunity involves the killing of pathogens or neoplastic cells by cytotoxic T lymphocytes. Other T lymphocytes perform helper and suppressor functions, regulating other immunological reactions. The most profound effects of HAHs on cell-mediated immunity occur upon exposure during growth and development of embryos and young animals. Such exposure reduces delayed-type hypersensitivity responses and *in vitro* proliferation of T lymphocytes, two important measures of cell-mediated immunity (Vos and Van Driel-Grootenhuis 1972; Sharma *et al.* 1978; Hinsdill *et al.* 1980; Thomas and Hinsdill 1980; Clark *et al.* 1981, 1983). Takagi *et al.* (1987) and Tomar *et al.* (1991) showed that PCBs suppressed helper T cell activity in mice. Effects of HAHs have been demonstrated at a number of steps in T cell development. Fine *et al.* (1990) demonstrated that TCDD reduced the activity of fetal liver and neonatal bone marrow prothymocytes in mice. TCDD also affects the maturation and selection of T lymphocytes in the developing thymus (Lundberg *et al.* 1990, Holladay *et al.* 1991), perhaps inhibiting the transition of $CD4^-CD8^+$ cells to $CD4^+CD8^+$ thymocytes (Blaylock *et al.* 1992). Greenlee *et al.* (1985) found that TCDD treatment *in vitro* reduced the ability of thymic epithelial cells to support proliferation of mitogen-stimulated lymphocytes. Neubert *et al.* (1991) showed a decrease in helper T cell numbers when lymphocytes from marmosets (*Callithrix jacchus*) and humans were cultured with TCDD, indicating direct effects on helper T cells. Similar reductions in helper T lymphocyte numbers have been observed in humans who have been exposed perinatally to PCBs, PCDDs (polychlorinated dibenzo-dioxins), and PCDFs

(polychlorinated dibenzo-furans) in arctic Quebec (Dewailly *et al.* 1993) and to TCDD in Times Beach, Missouri (Smoger *et al.* 1993). At Times Beach these effects persisted at least 10 years after the time period of highest exposure.

Hence, HAHs suppress a number of important immunological mechanisms, including antibody and cell-mediated responses. Given these effects on immune function, it is not surprising that laboratory animals exposed to HAHs have a reduced ability to combat infections. Halogenated aromatic hydrocarbons decrease resistance of laboratory mammals and birds to challenges with bacteria (Thomas and Hinsdill 1978, Vos et *al.* 1978, Hinsdill *et al.* 1980), viruses (Friend and Trainer 1970, Imanishi *et al.* 1980, Vos and Luster 1989, House *et al.* 1990), and protozoan parasites (Vos and Luster 1989). Children who have been exposed perinatally to PCBs, PCDDs, and PCDFs in arctic Quebec experienced an increased incidence of middle ear infections during the first year of life (Dewailly *et al.* 1993).

HAHs may have contributed to recent epizootics in marine mammals exposed to high concentrations of organochlorine pollutants. The disease outbreaks have occurred in beluga whales (*Delphinapterus leucas*) in the St. Lawrence Estuary (Martineau *et al.* 1988), California sea lions (*Zalophus californianus*) on San Miguel Island (Gilmartin *et al.* 1976, Barinaga 1988), common seals (*Phoca vitulina*) in Europe (McGourty 1988), and bottlenose dolphins (*Tursiops truncatus*) in the Atlantic Ocean (Lahvis *et al.* 1995). Brouwer *et al.* (1989) fed PCB-contaminated fish from the Wadden Sea to common seals and found that these seals had lower plasma retinol, triiodothyronine, and total and free thyroxine as compared to seals fed relatively uncontaminated fish from the northeast Atlantic. They hypothesized that, since retinol is important for immune function, the outbreaks of viral disease in European seals could have been caused by PCB-induced

retinol depletion. Several recent studies have lent support to this hypothesis. Harbor seals fed organochlorine-contaminated herring from the Baltic Sea had altered white blood cell counts and immune function (DeSwaart *et al.* 1994, Ross *et al.* 1995). Lahvis *et al.* (1995) found that T-cell immunity was inversely related to PCB and DDE contamination in male bottlenose dolphins from the Atlantic Ocean.

Many immunotoxic effects of HAHs are caused by coplanar congeners in animals that express the Ah receptor (Silkworth and Grabstein 1982, Silkworth *et al.* 1984, Nagarkatti *et al.* 1984, Safe 1984, Luster *et al.* 1987, Vos and Luster 1989, Kerkvliet *et al.* 1990a,b). Such effects are less pronounced when coplanar HAHs are administered to animals that lack the Ah receptor and when noncoplanar HAHs are administered to animals that possess the Ah receptor. However, other studies have suggested that HAHs can suppress humoral immune function via a mechanism that is independent of the Ah receptor (Kerkvliet *et al.* 1990a,b; Davis and Safe 1991; Morris *et al.* 1992). Field evidence suggests that coplanar HAHs acting via the Ah receptor are associated with reproductive problems and various pathologies in Great Lakes birds (Tillitt *et al.* 1989, 1992; Ecological Research Services 1991), and it is probable that immunosuppression is occurring since it also arises, at least in part, by an Ah-receptor mechanism.

Rationale for Conducting this Study

Fish-eating birds such as herring gulls, Caspian terns, and double-crested cormorants serve as "sentinel" or bioindicator species and provide a good opportunity for studying effects of contaminants on immune systems. Their colonial nesting behavior allows access to large numbers of birds and potentially increases the exposure of individuals to pathogenic microorganisms. Their high position in the food web exposes

them to elevated concentrations of HAHs and other persistent, fat-soluble chemicals. Biochemical and physiological indices have shown that many fish-eating birds, especially double-crested cormorants and Caspian terns, have been and continue to be impacted by pollutants in the Great Lakes, and they also may be experiencing immunosuppression. At highly contaminated sites in the Great Lakes, double-crested cormorants have an abnormally high incidence of eye infections (Ecological Research Services 1991). Caspian terns from highly contaminated U.S. colonies have lower rates of recruitment into the breeding population than those from less contaminated Canadian colonies (Ludwig 1979, Mora *et al.* 1993), suggesting contaminant-induced population effects possibly caused by immunosuppression. The occurrence of contaminant-induced immunosuppression has been suggested in herring gulls and common terns in contaminated sites in the Great Lakes (Colborn *et al.* 1990).

Although there is firm laboratory evidence for immunosuppression caused by a variety of contaminants, especially HAHs, very little effort has been directed to the rigorous study of immunological suppression in free-living animals. Recent studies have documented a number of pathologies in Great Lakes birds that are associated with coplanar PCBs and other TCDD congeners--precisely the congeners that have been shown to be powerful immunotoxins in laboratory studies. Hence, the effects of HAHs and other contaminants on the immune systems of Great Lakes birds warrant more rigorous study, especially since immunosuppression could have potentially drastic effects on waterbird population dynamics (i.e., reduced population growth or outright population declines associated with increased susceptibility to disease). Also, humans consuming Great Lakes fish may encounter similar health problems. This study was conducted in concert with

other biomonitoring efforts and provided the opportunity to test the effectiveness of using various immunological parameters as biomarkers for effects of contaminants in wildlife.

Objectives

The major objective of this project was to determine whether immune function is suppressed in herring gulls and Caspian terns exposed to contaminants, especially HAHs, in the Great Lakes. Understanding the effects of contaminants on individual survival is fundamental to predicting the effects on populations. The objectives stated here, while deliberately tightly focused, were proposed with a long-term goal of their application in understanding effects on population dynamics. To achieve the overall objective, a number of more specific objectives were set.

1. To determine whether the immune systems of herring gulls and Caspian terns are impaired by persistent environmental contaminants in the Great Lakes.

    A. To quantify general immunological and hematological parameters in these fish-eating birds that are exposed to different types and magnitudes of environmental contamination.

    B. To determine whether there are alterations in humoral and T cell-mediated immune function in fish-eating birds exposed to varying degrees of environmental contamination.

C. To evaluate potential immunotoxic effects using ecoepidemiological criteria.

2. To identify the contaminants (e.g., PCBs, TCDD, DDT) that are most likely responsible for immunosuppression in fish-eating birds in the Great Lakes.

3. To evaluate the use of various immunological tests as biomarkers for contaminant exposure in wild birds.

4. To quantify effects of contaminants on other physiological variables that may be related to immune function, including plasma concentrations of retinoids and thyroid hormones.

Ecoepidemiology and Risk Assessment

Given the rationale for conducting this study and the objectives stated above, it is important to consider the criteria by which to judge whether there is a causal relationship between contaminants and immunosuppression in Great Lakes fish-eating birds. Ecotoxicological studies can employ a combination of hypothesis testing and inductive reasoning to illustrate such relationships. Ecotoxicological studies can draw on a large data base of biological effects that have been demonstrated by hypothesis testing and manipulative experiments conducted under laboratory conditions. This laboratory-generated data base does not exist for many other types of ecological research. In some situations, toxicologists can conduct true manipulative experiments in the field (i.e., by introducing a contaminant to a study area and comparing effects on treated vs. untreated

areas). However, such a manipulative approach was not possible for the present study. However, specific hypotheses were tested by choosing appropriate study sites (i.e., by comparing reference sites with sites of various degrees of contamination).

Fox (1991) reviewed criteria for demonstrating causal relationships in ecotoxicological research. These criteria are drawn from principles of classical epidemiology. Fox defines ecoepidemiology as "the study of the ecological effects that are prevalent in certain localities or among certain population groups, communities, and ecosystems and their potential causes" (p. 362). For epidemiologists, establishing causation does not require that a factor is a necessary and sufficient condition to produce an effect. Rather, causal associations imply that a factor is part of a complex that increases the probability of an effect, and that reducing the factor reduces the probability of the effect. Causal relationships in ecoepidemiology can be demonstrated by weighing seven criteria: probability, chronological relationship, strength of association, specificity, consistency of association upon replication, predictive performance, and coherence. The following discussion briefly describes how the present study used these criteria to examine the general hypothesis that environmental contaminants, especially HAHs, are associated with immunosuppression in fish-eating birds of the Great Lakes.

Probability

Probability, or statistical significance, is one criterion for establishing a causal association. However, Fox (1991) notes that lack of a *statistically* significant relationship may not necessitate the rejection of an association, especially if there are other reasons for accepting a *biologically* significant relationship. In the present study, relationships between contaminants and immune function were examined by a variety of statistical methods,

including standard analysis of variance (ANOVA) or covariance (ANCOVA), the nonparametric Jonckheere test for ordered alternatives, and correlation. Following ANOVA analysis, multiple comparison techniques were used to compare immune function at contaminated sites to that at reference sites.

Strength of Association

Strength of association generally refers to the relative risk of exhibiting a given effect in a population exposed to the putative causal factor as compared to an unexposed population. It also can be thought of as "the degree to which the supposed cause and outcome coincide in their distribution" (Fox 1991:367). Strength of association often can be determined by examining the incidence and magnitude of a given effect across a gradient of exposures. The strong association of HAH contamination with the incidence of specific effects has been an important factor in linking effects to HAHs in Great Lakes wildlife. The expression of the biological activity of complex mixtures of HAHs as TCDD-equivalents has increased the strength of statistical relationships between HAHs and biological effects. For example, Tillitt *et al.* (1992) found an inverse correlation between total PCB concentrations and reproductive success ($r=-0.56$, $p=0.045$), but the correlation between TCDD-equivalents and reproductive success was much stronger ($r=-0.84$, $p=0.003$).

Specificity

Specificity can be examined from two perspectives. First, is the effect of interest produced *only* by the putative cause? In the present study, the fact that so many factors (nutrition, stressors, environmental factors) could have influenced immune function

weakened the usefulness of this criterion. However, if an association between contaminants and immunosuppression can be demonstrated even in the presence of these confounding factors, the argument for a causal relationship may in fact be strengthened. A second way to examine specificity is to ask whether the putative causal factor usually produces a given effect, even if it is not the only cause for that effect. Because immunosuppression is one of the most characteristic symptoms of HAH exposure, the second form of the specificity criterion was an important consideration in the present study.

Consistency Upon Replication

Replication is very important for the design of any study. Although replicate study sites for the present study could not be chosen randomly, sites were chosen to replicate degrees of contamination. Study sites were chosen so that there were at least two low and two high contamination sites for each species. Herring gulls were studied at reference sites outside the Great Lakes. Furthermore, Fox writes, "In ecoepidemiology, the occurrence of an association in more than one species and species population is very strong evidence for causation" (Fox 1991:368). This study attempted to replicate immunosuppressive effects by studying two species of fish-eating birds. Furthermore, immunological function was measured during three or four consecutive years at some sites, providing an opportunity to replicate among years.

Coherence

Like specificity, coherence can be examined in several ways. First, how does the relationship fit into the current body of biological knowledge? For this project, the wealth of information on the immunotoxic effects of HAHs in laboratory animals allowed adequate

evaluation of this criterion. Secondly, coherence refers to the presence of a dose-response relationship. Sampling across a contaminant gradient provided dose-response information. However, it is important to note that not all dose-response relationships must be linear--some may exhibit thresholds or sigmoidal or parabolic shapes. In the present study, the Jonckheere test for ordered alternatives was used to examine the relationship between immunological variables and organochlorine contamination, including TCDD-equivalents. The Jonckheere test detects monotonic trends in the response but does not prejudge the shape of the response (i.e., linear, exponential, etc.).

Time Order or Chronological Relationship

To demonstrate a causal relationship, the effect must be absent before the introduction of the causal factor. Unfortunately, there are no data on immune function in Great Lakes fish-eating birds before the era of pollution. However, in this study, birds were exposed to environmental contaminants before immunological variables were measured.

Predictive Performance

Based on laboratory data, a number of basic predictions were made regarding the effects of HAHs on immune function in fish-eating birds at sites with various degrees of contamination. The fulfillment of these predictions was a strong argument for a causal relationship between contaminants and immunosuppression. The Jonckheere test tested specific hypotheses that were predicted from experimental evidence and exposure data. The data gathered by this project hopefully will allow the formulation of more specific predictions that can be tested in the future.

Strategy for Statistical Analyses

The main strategy for statistical analysis was shaped by two factors: 1) the importance of testing specific hypotheses regarding the relationships between immunological variables and organochlorine contaminants, and 2) the great expense of congener-specific PCB analysis. Because of this expense, contaminant analyses could not be performed on tissues from individual birds. Instead, twelve eggs from each site or portions of the liver from each bird were pooled and homogenized. Contaminant concentrations were then measured in the pooled sample from each site. This approach allowed the sites to be ranked in order of contamination for various chemicals but did not allow individual birds to be ranked in the same manner.

Because contaminant concentrations were not measured in individual birds, standard regression or correlation techniques were not appropriate. However, the Jonckheere test for ordered alternatives provided an effective way to use the contaminant rankings by site. The null hypothesis for this nonparametric test states that there is no difference among the central tendencies from different sites (H_O: $\mu_1 = \mu_2 = \mu_3 = ... = \mu_n$). The alternative hypothesis is that there is a monotonic trend in the data based on *a priori* information (H_A: $\mu_1 \geq \mu_2 \geq \mu_3 ... \mu_n$, where at least one of the inequalities is a strict inequality). In this study, laboratory studies or other field data were used to predict the effects of various contaminants on each response variable whenever possible. If the direction of the trend for a variable could not be easily predicted from laboratory data, a two-way test was performed by running the Jonckheere test in both directions and doubling the P-value for the most significant trend. The following scale was used to describe the strength of the evidence against the null hypothesis: P>0.2, little or no evidence;

$0.1<P<0.2$, very weak evidence; $0.05<P<0.1$, marginal evidence; $0.01<P<0.05$, moderate evidence; $0.001<P<0.01$, strong evidence; and $P<0.001$, very strong evidence.

Before the Jonckheere test was performed, ANOVA or ANCOVA was used to determine whether the response variable was influenced by factors such as site, year, sex, or any interactions. If the Jonckheere tests did not show any statistically significant trends, then ANOVA or ANCOVA followed by Duncan's multiple range test was used to look for differences among sites. Alpha was set at 0.05 for all judgements about pooling data across years or sexes.

Chapter 2. Immunological and Hematological Biomarkers for Environmental Contaminants in Herring Gulls (*Larus argentatus*) of the Great Lakes

Summary

This study explored the feasibility of using white blood cell counts and packed red cell volume (PCV) as immunological and hematological biomarkers for assessing the health of herring gulls, especially in relation to persistent environmental contaminants. If important structural components of the immunological and hematological systems are disrupted, it is likely that physiological functions also will be compromised. This study sought to determine whether the immunological and hematological systems of herring gull chicks and adults are affected by environmental contaminants, especially halogenated aromatic hydrocarbons (HAHs), in the Great Lakes. In herring gull adults, total white blood cell counts were conducted at seven sites and differential counts at twelve sites. In adults, there was very strong evidence that total leukocyte and total heterophil numbers increased as liver concentrations of DDE increased and as liver activity of ethoxyresorufin-O-deethylase (EROD) decreased. Total lymphocytes increased as liver PCB concentrations increased. In adults, there was very strong evidence that the heterophil to lymphocyte ratio decreased as liver EROD increased. In herring gull chicks, total white blood cell counts were conducted at three sites and differential counts at ten sites. There was a very strong positive relationship between the heterophil to lymphocyte ratio and HG-TEQs (dioxin toxicity equivalents as calculated from herring gull-specific toxicity equivalency factors).

PCV was influenced by contaminants in both herring gull adults and chicks, although the magnitudes of these changes were not great. The values of the hematological variables measured in this study were similar to those from other studies on herring gulls and related species. This study showed alterations in white blood cell counts in Great Lakes herring gulls that were associated with environmental contaminants. Hematological variables are effective, economical, nondestructive biomarkers for assessing the health of wild birds.

Introduction

Biomarkers are biochemical, physiological, or histological changes that indicate effects of, or exposure, to toxic chemicals. The development of biomarkers for immunotoxic effects presents difficult problems because many laboratory assays for immune function involve sophisticated techniques such as cell culture that are not easily conducted during field investigations. This study explored the feasibility of using simpler structural measurements, namely white blood cell counts and packed red cell volume (PCV), as immunological and hematological biomarkers for assessing the health of wild birds. The connection between structure and function is one of the unifying themes in biology. White blood cell counts have been included as Tier I tests for screening chemicals for immunotoxic potential in the National Toxicology Program (Luster *et al.* 1988b, 1994). Although these simple tests often are less sensitive than direct tests of immune function, a significant alteration in white blood cell numbers usually is associated with abnormal immune function.

The immunosuppressive effects of halogenated aromatic hydrocarbons (HAHs), which include polychlorinated biphenyls (PCBs), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

(TCDD), and 2,3,7,8-tetrachlorodibenzo-*p*-furan (TCDF), have been established by numerous laboratory studies. While most laboratory studies have used sophisticated assays for immune function, several studies have found that HAHs can change the number of circulating lymphocytes (Vos and Van-Driel Grootenhuis 1972, Vos and Luster 1989) and decreased PCV (Hinsdill *et al.* 1980). Low doses of HAHs characteristically suppress T cell-mediated immunity, especially in young animals. T lymphocytes mature in the thymus gland and are responsible for regulating immune responses and attacking virus-infected cells and malignant cells. In developing animals, low levels of HAHs cause thymic atrophy (Vos and Van Driel-Grootenhuis 1972, Silkworth and Grabstein 1982, Nikolaidis *et al.* 1988a,b, Vos and Luster 1989, Andersson *et al.* 1991). Toxic effects have been observed at many stages of T lymphocyte development, including the prothymocyte stage in the bone marrow (Fine *et al.* 1990), thymocyte selection in the thymus (Greenlee *et al.* 1985, Lundberg *et al.* 1990, Holladay *et al.* 1991, Blaylock *et al.* 1992), and the mature T lymphocyte stage in the blood (Neubert *et al.* 1991). As a result, a number of T cell functions are suppressed (Vos and Van Driel-Grootenhuis 1972; Sharma *et al.* 1978; Hinsdill *et al.* 1980; Thomas and Hinsdill 1980; Clark *et al.* 1981, 1983; Takagi *et al.* 1987; Tomar *et al.* 1991). B lymphocytes develop in the bursa of Fabricius in birds and the bone marrow in most mammals. B lymphocytes respond to foreign antigens by producing specific antibodies, which help destroy invading microorganisms. High doses of HAHs suppress antibody production in laboratory animals (Sharma *et al.* 1978; Thomas and Hinsdill 1978; Hinsdill *et al.* 1980; Clark *et al.* 1981, 1983; Silkworth and Grabstein 1982; Silkworth *et al.* 1984; Luster *et al.* 1988a, House *et al.* 1990; Kerkvliet *et al.* 1990b). HAHs appear to exert their toxic effects on the immune system primarily through *Ah*-receptor-dependent mechanisms (Silkworth and Grabstein 1982, Silkworth *et*

al. 1984, Nagarkatti *et al.* 1984, Safe 1984, Luster *et al.* 1987, Vos and Luster 1989, Kerkvliet *et al.* 1990a,b), although mechanisms independent of the *Ah*-receptor also are involved (Kerkvliet *et al.* 1990a,b, Davis and Safe 1991, Morris *et al.* 1992). In laboratory mammals and birds, these immunosuppressive effects of HAHs increase susceptibility to bacteria (Thomas and Hinsdill 1978, Vos et *al.* 1978, Hinsdill *et al.* 1980), viruses (Friend and Trainer 1970, Imanishi *et al.* 1980, Vos and Luster 1989, House *et al.* 1990), and protozoan parasites (Vos and Luster 1989).

HAHs may have contributed to epizootics in marine mammals exposed to high concentrations of organochlorine pollutants. The disease outbreaks have occurred in beluga whales (*Delphinapterus leucas*) in the St. Lawrence Estuary (Martineau *et al.* 1988), California sea lions (*Zalophus californianus*) on San Miguel Island (Gilmartin *et al.* 1976, Barinaga 1988), common seals (*Phoca vitulina*) in Europe (McGourty 1988), and bottlenose dolphins (*Tursiops truncatus*) in the Atlantic Ocean (Lahvis *et al.* 1995). Brouwer *et al.* (1989) fed PCB-contaminated fish from the Wadden Sea to common seals and found that these seals had lower plasma retinol, triiodothyronine, and total and free thyroxine concentrations. They hypothesized that since retinol is important for immune function, the outbreaks of viral disease in European seals could have been caused by PCB-induced retinol depletion. Several recent studies have supported this hypothesis. Harbor seals fed organochlorine-contaminated herring from the Baltic Sea had altered white blood cell counts and immune function (DeSwaart *et al.* 1994, Ross *et al.* 1995). Lahvis *et al.* (1995) found that T-cell immunity was inversely related to PCB and DDE contamination in male bottlenose dolphins from the Atlantic Ocean.

Fish-eating colonial waterbirds are excellent candidate sentinel species for immunotoxicological assessments in the Great Lakes basin. Their high position in the food

web exposes them to high concentrations of contaminants that biomagnify, such as HAHs. Numerous studies have documented significant reproductive, developmental, and behavioral impacts of these chemicals (Peakall *et al.* 1980; Mineau *et al.* 1984; Fox and Weseloh 1987; Peakall and Fox 1987; Gilbertson 1988, 1989; Peakall 1988; Colborn *et al.* 1990; Fox *et al.* 1991; Gilbertson *et al.* 1991; Fox 1993). At sites that are highly contaminated with PCBs, colonial waterbirds have experienced severe developmental effects that include high embryonic mortality, subcutaneous, pericardial, and peritoneal edema, congenital deformities, growth retardation, hepatomegaly, liver necrosis, and liver porphyria (Gilbertson 1989). Gilbertson *et al.* (1991) named this set of symptoms Great Lakes embryo mortality, edema, and deformities syndrome (GLEMEDS). Furthermore, Caspian terns hatched at highly contaminated Great Lakes sites show a low recruitment rate into the breeding population (Ludwig 1979, Mora *et al.* 1993). Immunotoxic effects of environmental contaminants could contribute to these impacts on populations. The dense nesting conditions of colonial waterbirds promote the transfer of infectious diseases among individuals, and histopathological analysis of apparently healthy and sick colonial waterbirds shows the presence of many infectious microorganisms and parasites. At highly contaminated sites in the Great Lakes, double-crested cormorants (*Phalacrocorax auritus*) have an abnormally high incidence of eye infections associated with *Pasteurella multocida* (Ecological Research Services 1991).

Several studies have attempted to use hematological variables to assess the health of wild and captive birds. Iturri (1974) found that PCV, red blood cell counts, and blood hemoglobin concentration displayed similar decreases in chickens fed various mixtures of PCBs. Rocke and Samuel (1991) reported that mallard ducks (*Anas platyrhynchos*) exposed to lead shot experienced decreases in white blood cell counts and PCV. Grasman

and Scanlon (1995) found that lead influenced differential white blood cell counts and decreased PCV in Japanese quail (*Coturnix coturnix*). Averbeck (1991, 1992) found that PCV, red blood cell count, and blood hemoglobin concentration showed similar decreases in sick herring gulls (*Larus argentatus*) and great black-backed gulls (*L. marinus*) as compared to healthy gulls. White blood cell counts did not differ between healthy and sick gulls. Leighton (1984) reported that ingestion of crude oil increased the heterophil to lymphocyte ratio in herring gull chicks.

This study sought to determine whether the immunological and hematological systems of herring gull chicks and adults are affected by persistent environmental contaminants, especially HAHs, in the Great Lakes. Severe organochlorine contamination might suppress leukocyte and erythrocyte counts and alter relative numbers of leukocyte types. Alternatively, immunosuppression caused by environmental contaminants or other factors might lead to chronic infections that would increase leukocyte counts. This study examined the usefulness of hematological biomarkers for monitoring effects of contaminants, and it compared these hematological biomarkers to several common biochemical biomarkers related to immune function and (or) contaminant exposure.

Materials and Methods

Sampling Design

Study sites were chosen across a wide range of organochlorine contamination (Table 2.1). Adult herring gulls were sampled from eleven sites within the Great Lakes (Fig. 2.1.A): 1) an officially unnamed island (Pump Station Island) near Jackfish Bay, northern Lake Superior; 2) Granite Island in Black Bay, north shore of Lake Superior; 3)

Gull Island in upper Green Bay, Lake Michigan; 4) Kidney Island in lower Green Bay, Lake Michigan; 5) Confined Disposal Facility in Saginaw Bay, Lake Huron; 6) Chantry Island, southeastern Lake Huron; 7) Fighting Island, in the Detroit River; 8) Middle Island, western Lake Erie; 9) Middle Sister Island, western Lake Erie; 10) Hamilton Harbour, western Lake Ontario; and 11) Scotch Bonnet Island, eastern Lake Ontario. Twenty-eight-day-old herring gull chicks were collected from nine Great Lakes sites (Fig. 2.1.B): 1) an officially unnamed island (Pump Station Island) near Jackfish Bay, northern Lake Superior; 2) Gull Island in Upper Green Bay, Lake Michigan; 3) Double Island in the North Channel of Lake Huron; 4) Little Charity Island in Saginaw Bay, Lake Huron; 5) Chantry Island, southeastern Lake Huron; 6) Monroe, western shore of Lake Erie; 7) Hamilton Harbour, western shore of Lake Ontario; 8) Scotch Bonnet Island, eastern Lake Ontario; and 9) Stracken Island, St. Lawrence River. For both ages of herring gulls, two reference sites outside the Great Lakes were sampled: Kent Island in the Bay of Fundy off Canada's Atlantic coast; and Pony Island, in the northern end of Lake Winnipeg, Manitoba. Total white blood cell counts were conducted at seven sites for adults and three sites for chicks. Differential white blood cell counts were not made on adults at Middle Sister Island and chicks from Monroe, Lake Erie. Adult gulls were sampled during 1993 at Middle Sister Island. Chicks were collected during 1992 at Double Island, Stracken Island, and Monroe. All other collections were made during the 1991 breeding season.

Adult herring gulls were captured by drop-trapping over nests during mid-incubation. Prior to trapping, eggs were floated to determine the buildup of air inside the egg and hence the approximate stage of incubation. Herring gull chicks were randomly selected and captured at the approximately 28 days of age. Approximately ten birds of each age were collected at each of the study sites listed above. For several sites, 15-20 birds

were collected to determine whether sample sizes larger than ten provide significantly greater sensitivity for biomonitoring. An effort was made to collect equal numbers of adult males and females from each site.

Hematology

A blood sample (approximately 11 ml) was drawn from the brachial vein using a 22 gauge needle and Vacutainer® tubes (Beckton Dickinson, Rutherford, NJ). Seven ml of blood were drawn into a tube containing dry sodium heparin and 4 ml into a tube containing dry potassium EDTA. Two blood smears were made quickly from the blood collected in EDTA. The heparinized blood was centrifuged immediately at 2575×g for five minutes, and the plasma was stored on liquid nitrogen for vitamin A and thyroxine analysis. The PCV was measured by centrifuging microhematocrit tubes filled with blood collected in EDTA at 5125×g for 5 minutes in a clinical centrifuge equipped with a hematocrit rotor.

The blood collected in EDTA was kept on ice until a total white blood cell count was completed, usually on the same day as collection. Dilutions were made with standard 101-1 erythrocyte pipettes in modified Natt and Herrick's diluent following the procedures of Gross (1984). Leukocytes were counted under a Neubauer hemacytometer at 400× magnification.

Blood smears were air-dried and fixed in methanol to preserve the cells until staining. Smears were stained with Wright stain (Accustain™, Sigma, St. Louis, MO) using 100% stain for 30 seconds and then diluting with an equal volume of distilled water for 90 seconds. Smears were rinsed with distilled water and allowed to air dry. Two hundred white blood cells were counted and classified using oil immersion microscopy at

1000× magnification. The total number of each type of white blood cell was determined by multiplying its percentage from the differential count by the total leukocyte count.

Dissection and Tissue Preservation

Before dissection, the body size of each gull was determined by measuring body mass, head length, bill depth, foot length, and, in chicks, wing chord (Fox *et al.* 1981). All chicks of appropriate size and all adults were sacrificed by decapitation using a guillotine and allowed to bleed out fully. The liver was quickly removed. The left lobe of the liver was later to be used for ethoxyresorufin-O-deethylase (EROD) analysis. This lobe was wrapped in high density polyethylene film, placed in 20 ml polyethylene scintillation vials, and preserved in liquid nitrogen within ten minutes after death. The concentrations of vitamin A, highly carboxylated porphyrin, and organochlorine contaminants were to be measured in the right lobe. Eight 1 gram samples of the right lobe of the liver were placed in 1 ml vials and preserved in liquid nitrogen for biomarker analysis. The remainder of the right lobe of the liver was placed in an acetone-hexane-rinsed glass jar and transported to the laboratory on wet ice before freezing at -20 °C prior to organochlorine analysis. The gonads, liver, adrenal glands, thyroid glands, and spleen of each bird were preserved in a neutral buffered formalin solution (10%). The thymus gland and bursa of Fabricius in chicks also were preserved in this fashion. In the laboratory, the thyroids, adrenals, spleen, thymus, and bursa were trimmed from associated connective tissue, blotted, and weighed to the nearest 0.01 g. Standard paraffin sections of the spleen, thymus, bursa, and liver were stained with hematoxylin and eosin and examined by avian histopathologists using standard histopathological techniques.

Biochemical Biomarkers

Vitamin A compounds in the liver were extracted from 0.3-0.5 g liver samples and dehydrated by grinding with anhydrous sodium sulphate. Powder equivalent to 0.20 g of liver was combined with 8-20 ng of retinyl acetate in 20 µl of 2-propanol as an internal standard. Retinoids were extracted in 10 ml of dichloromethane:methanol (1:9) by repeated shaking and liberation of pressure for 5 minutes. The extract was centrifuged at 600 rpm and 10 °C for 10 minutes. An aliquot of the supernatant was filtered through a 0.20 or 0.45 µm filter disk. The final extract (20 µl) was analyzed by nonaqueous reverse-phase HPLC.

Plasma retinol was extracted from 100 µl of plasma after the addition of 75 ng of retinyl acetate in 15 µl of 2-propanol as an internal standard. Retinol-protein complexes were dissociated by vigorous shaking after the addition of 200 µl of acetonitrile. The retinol was extracted with successive 4 and 1 ml volumes of hexane. After shaking for 5 minutes, the organic and aqueous phases were separated by centrifugation at 1500 rpm for 5 minutes at 4 °C. The organic phase was dried under nitrogen, reconstituted with 500µl of methanol, and filtered through a 0.2 µm PVDF filter disk.

The liver and plasma extracts were analyzed for vitamin A compounds by reverse-phase HPLC using an ODS spheri-5 guard column (Brownlee) and a 15 cm long, 5 µm ODS Zorbax (DuPont) analytical column. For the liver extract, the methanol and dichloromethane were used to resolve the retinoids in less than 10 minutes. Methanol was used to separate the retinoids in the plasma extract in less than 6 minutes. Either fluorescence (ex: 336 nm; em: 480 nm) or UV-visible (326 nm) was used to detect the retinoids. The detection limits were 5 µg/L retinol in plasma, 0.4 µg/g retinol in liver, and 1.2 µg/g retinyl palmitate in liver.

The left lobe of each liver was homogenized, and microsomes were isolated following the procedure of Pyykko (1983). The catalytic activity of EROD in the liver was determined using the methods of Kennedy and Jones (1994) optimized for herring gull liver microsomes. Highly carboxylated porphyrins were measured in liver tissue following the procedure of Kennedy and James (1993). In plasma samples from adults, total thyroxine was measured using a solid phase enzyme assay (veterinary modification of the EZ Bead T4 Test, Immunotech Corp, Boston, MA).

Organochlorine Analyses

The 10-20 individual liver samples collected for each age and site were pooled for organochlorine analysis by the analytical services laboratory at the National Wildlife Research Centre of the Canadian Wildlife Service following the methods of Peakall *et al.* (1986). PCB residues are reported as the sum of the following 42 PCB congeners: IUPAC nos. 28, 31, 42, 44, 49, 52, 60, 64, 66, 70, 74, 87, 97, 99, 101, 105, 110, 118, 128, 129, 137, 138, 141, 146, 149, 151, 153, 158, 170, 171, 172, 174, 180, 182, 183, 185, 194, 195, 200, 201, 203, and 206.

Nonortho PCB congeners (IUPAC nos. 37,77,126, and 169) and all 2,3,7,8-substituted polychlorinated dibenzo-dioxin (PCDD) and polychlorinated dibenzo-furan (PCDF) congeners also were measured in pooled liver samples by the analytical services laboratory of the National Wildlife Research Centre of the Canadian Wildlife Service. Pooled liver samples were dried with anhydrous sodium sulphate and ground into a fine powder. An open chromatographic column wet-packed with multiple absorbents (anhydrous sodium sulphate, deactivated silica, sulfuric acid on silica, activated silica, and sodium hydroxide on silica) was used for the initial extraction and cleanup. The sample

was spiked with a ^{13}C PCDD mixture (Cambridge Isotope Laboratories) and a ^{13}C PCB 77, 126, and 169 mixture (Wellington Isotope Laboratories) to determine the degree of analyte loss during sample workup. The column was eluted using 1:1 dichloromethane/hexane, and the eluted extract was concentrated by evaporation. A liquid chromatograph (FMS Systems Inc.) with a carbon column was used for further cleanup and trace enrichment. The carbon column was flushed with dichloromethane and backflushed with toluene. The eluent was concentrated by evaporation, and the solvent was changed to hexane. After concentration by evaporation, the extract was cleaned up and separated on a deactivated Florisil column wet packed in hexane. The column was first eluted with 1:20 dichloromethane/hexane for nonortho PCB analysis. The column was then eluted with dichloromethane to produce a second fraction that was cleaned up on a wet packed, activated basic alumina column (Fisher Scientific). The alumina column was eluted with 1:50 dichloromethane:hexane to produce a fraction containing residual PCBs and other organochlorines. The column was then eluted with 1:1 dichloromethane:hexane to produce a fraction for PCDD/PCDF analysis. A Hewlett-Packard 5971A GC/MSD was used to separate and quantify nonortho PCB, PCDD, and PCDF congeners. Detection limits were 75 pg/g for nonortho PCB congeners and approximately 0.5-25 pg/g for PCDD and PCDF congeners.

Because different HAH congeners have different relative toxicities, the total biological activity of a complex mixture of congeners cannot be estimated by adding the concentrations of the individual congeners. Furthermore, the relative toxicities of different congeners vary from species to species. Kennedy *et al.* (1994) used *in vitro* induction of EROD activity in primary hepatocyte cultures from 26 day old herring gull embryos to compare the relative toxicities of TCDD, TCDF, and various PCBs. Based on the EC_{50} for

EROD induction, different HAH congeners could be compared to TCDD, the most toxic congener. The following herring gull-specific toxicity equivalency factors (TEFs) were generated: TCDD = 1.0; TCDF = 0.9; PCB congener # 169 = 0.07, PCB congener #126 = 0.06; PCB congeners # 77, #105, #118=0. Multiplying the concentration of each congener by its TEF and then summing the products gave an estimate of the total dioxin-like toxicity of the mixture for herring gulls. This estimate was called HG-TEQs, which stands for herring gull-specific TCDD-equivalents.

Statistical Analyses

The primary goal of this investigation was to determine whether there was an association between the degree of organochlorine contamination and immunological variables in fish-eating birds of the Great Lakes. The purpose was to determine whether intercolony differences in immunological variables could be explained by exposure-response relationships. Statistical methods were needed to determine the probability that the spatial patterns in response variables were associated with different pollutants as opposed to being caused by random events. The purpose was not to show what percent of variability in immunological responses could be explained statistically by particular chemicals. Because detailed organochlorine analyses are expensive and require large volumes of blood, contaminant concentrations were not measured in individual chicks. However, chemical analysis of pooled liver samples from each site allowed the sites to be ranked on the basis of concentrations of different contaminants.

Statistical methods were chosen to meet this primary goal of associating immunological variables with pollutants and to work within the constraints of pooled chemical analyses. Regression analysis was not appropriate because of its emphasis on

explaining variability using linear models and its need for chemical analyses on individual birds. Although analysis of variance (ANOVA) could detect spatial differences in immune responses among sites, it was not appropriate because it could not be used to look for associations across gradients of contamination.

The Jonckheere test for ordered alternatives was used as the primary statistical method of testing specific hypotheses about contaminant-associated immunosuppression (Hollander and Wolfe 1973). This test fit the purpose and design of the study as well as the constraints on chemical analysis. The null hypothesis for this nonparametric measure of exposure-response states that there is no difference among the central tendencies from different sites (H_O: $\mu_1 = \mu_2 = \mu_3 = ... = \mu_n$). The alternative hypothesis is that there is a monotonic trend (not necessarily linear) in the data based on *a priori* information (H_A: $\mu_1 \geq \mu_2 \geq \mu_3 ... \mu_n$, where at least one of the inequalities is a strict inequality). The direction of the trends for PCV and white blood cell counts could not be easily predicted from laboratory data, so a two-way test was performed by running the Jonckheere test in both directions and doubling the P-value for the most significant trend. Severe immunosuppression could have caused leukopenia, or recurring infections caused by immunosuppression could have caused leukocytosis. The following variables were used to order the study sites: liver concentrations of total PCBs, DDE, and calculated HG-TEQs; and liver EROD activity. The Jonckheere test was performed using a custom-written FORTRAN program (B. Collins, Senior Statistician, Canadian Wildlife Service) using the algorithm provided by Gibbons (1971). This program determined probability values based on 5000 random permutations of the data set being analyzed. These probability values were very similar to those found by the large sample normal approximation for the Jonckheere test (Hollander and Wolfe 1973).

While the Jonckheere test was the primary statistical test, analysis of variance (ANOVA) was used to for two purposes. First, ANOVA was used to determine whether the response variable was influenced by sex or a sex × site interaction in adults. If there was no statistically significant sex or a sex × site interaction effect ($P<0.05$), then data were pooled across years for the Jonckheere test. Second, if the Jonckheere test revealed no significant relationships with contaminants, then ANOVA was used to look for differences among sites. For ANOVA analyses, the heterophil/lymphocyte ratios were transformed ($\log_{10}$) to satisfy assumptions of homogenous variances and normality (Zar 1984). This transformation also was used for the Jonckheere and correlation tests. Organ masses were converted to percent of body mass before statistical analyses.

Relationships between the various biomarkers were detected using Pearson's correlation analysis. Statistically significant correlations ($P<0.05$) were reported only if the absolute value of the correlation coefficient (r) was greater than 0.3.

Results

Organochlorine Concentrations and EROD Activity

Organochlorine contaminants reached higher concentrations in herring gull adults than chicks (Tables 2.1 and 2.2). Minimum concentrations were 15.1× greater in adults for PCBs, 4.9× greater for HG-TEQs, and 19.7× greater for DDE. Maximum concentrations were 4.7× greater in adults for PCBs, 9.4× greater for HG-TEQs, and 2.7× greater for DDE. Although concentrations were generally higher in adults, the gradient from least to most contaminated sites was greater in chicks for PCBs and DDE. From the least to most contaminated herring gull chick livers, there was a 42.1× increase in PCBs, a 26.4×

increase in HG-TEQs, and an 80.3× increase in DDE. In adults, there was a 13.2× increase in PCBs, a 50.4× increase in HG-TEQs, and a 12.5× increase in DDE. PCB, HG-TEQ, and DDE concentrations in livers of chicks from Upper Green Bay were much higher than those from Saginaw Bay, which was the next most contaminated site. Livers from adults from Middle Island in Lake Erie had an extremely high value for calculated HG-TEQs. This value was driven by a high concentration of PCB congener #126.

EROD activity was higher in adults than in chicks (1.8× for minimum values, 1.5× for maximum values). Maximum induction for both chicks and adults occurred at Hamilton Harbour in Lake Ontario. From the lowest to highest values, chicks showed an increase of 11.6× and adults showed an increase of 9.5×. Over the entire exposure range, mean EROD activity was not correlated with PCBs (r_{11}=-0.12, P=0.72 for chicks, r_{13}=-0.06, P=0.84). However, EROD activity was positively correlated with PCB concentration at lower exposures. Mean EROD activity increased with PCBs up to 1.0 μg/g in chicks (r_7=0.92, P=0.004) and up to 10 μg/g in adults(r_6=0.90, P=0.015). Above these concentrations, mean EROD decreased in chicks and was variable in adults.

Immunological and Hematological Biomarkers

Total white blood cell counts were associated with spatial variation in environmental contaminants in adult herring gulls but not in chicks (Tables 2.3 and 2.4). In adults, there was little or no evidence that sex influenced any of the white blood cell variables. There was very strong evidence that total leukocytes and total heterophils increased as liver concentrations of DDE increased and as liver EROD decreased (Table 2.4; Fig. 2.2). Total lymphocytes increased as liver PCB concentrations increased (Table 2.4; Fig. 2.3). Total white blood cell counts were conducted on chicks at three sites. There was no evidence

that contaminants influenced numbers of total leukocytes, total heterophils, or total lymphocytes (Table 2.3). There was no evidence that these parameters differed among sites ($F_{2,22}$=0.029, P=0.97 for total leukocytes; $F_{2,22}$=0.035, P=0.97 for total heterophils; $F_{2,22}$=0.0.25, P=0.78; Table 2.5).

Differential white blood cell counts differed among sites in both chicks and adults. In chicks, there was a very strong positive relationship between HG-TEQs and the heterophil to lymphocyte ratio (Table 2.3, Fig. 2.4). In adults, there was very strong evidence that the heterophil to lymphocyte ratio decreased as liver EROD increased (Table 2.4; Fig. 2.5).

PCV was influenced by contaminants in both herring gull adults and chicks, although the magnitudes of these effects were not great (Tables 2.5 and 2.6). In chicks, there was strong evidence that PCV increased with EROD (Table 2.3). However, all but two sites had PCVs in the range of 29-33%. Pony Island on Lake Winnipeg was lower at 27% and Double Island on Lake Huron was higher at 36%. In adults, there was moderate evidence that female gulls had lower PCVs than males ($F_{1,134}$=6.14, P=0.014). However, the magnitude of this effect was small: across all sites the mean PCV was 42.5% for females and 43.5% for males. When data for males and females were pooled, there was only moderate evidence that PCV decreased as DDE increased (Tables 2.4 and 2.6).

Relationships among Hematological Biomarkers, Biochemical Biomarkers, and Organ Masses

Pearson's correlation analysis was used to explore relationships among biomarkers (Table 2.7). The objective was to determine whether there were any associations between

different immunological and hematological parameters and whether any biochemical biomarkers could serve as surrogates for immunological biomarkers.

Hematological variables showed few correlations with biochemical biomarkers that indicate effects of, or exposure to, HAHs. In adult herring gulls, the heterophil to lymphocyte ratio ($P<0.001$) and total heterophils ($P=0.009$) showed negative correlations with porphyrins. Total white blood cell numbers were not correlated with porphyrins ($P=0.18$). Conversely, in herring gull chicks there was a strong positive correlation between highly carboxylated porphyrins and both total leukocyte and total heterophil counts ($P<0.001$ for both correlations). There was only marginal evidence for a correlation between the heterophil to lymphocyte ratio and porphyrins ($P=0.058$). There were no statistically significant correlations with other biochemical biomarkers (plasma thyroxine or plasma or liver vitamin A compounds) in either chicks or adults.

In herring gull chicks, as body size increased, the relative number of heterophils to lymphocytes decreased without affecting the total white blood cell count, although the total counts were conducted at only three sites. The heterophil to lymphocyte ratio was negatively correlated with body mass ($P=0.006$) and wing chord ($P<0.001$). PCV increased with body mass in chicks ($P<0.001$). No associations were evident among body size and hematological variables in adults.

In herring gull chicks, various white blood cell variables were correlated with the masses of some immune and endocrine organs. A single chick from Upper Green Bay had a bursa three times larger than any other chick. When this bird was excluded from analyses, there was no evidence for any correlations between bursa mass and total white blood cells ($P=0.74$) or total heterophils ($P=0.77$). Total white blood cell counts were not correlated with thymus mass ($P=0.16$). Lymphocyte counts (total or percent) showed no

correlation to bursa or thymus masses. The heterophil to lymphocyte ratio was negatively correlated with the thyroid mass (P=0.002). Thyroid mass was not correlated with total white blood cell counts (P=0.33). Adrenal mass was not correlated with any hematological variables in either chicks or adults.

Discussion

White blood cells provide important defenses against invading microorganisms and parasites that have penetrated the epithelial barriers. White blood cells can carry out their functions in the blood and associated lymphoid tissues, and they can move into infected or inflamed tissues. Nonspecific defenses include the phagocytic and cytotoxic activities of heterophils (in birds) and neutrophils (in mammals). Monocytes are white blood cells that turn into phagocytic macrophages when they move into the tissues. Natural killer (NK) cells are cytotoxic to invading microorganisms. Other white blood cells react only against those pathogens that specifically fit receptors on their cell surface. B lymphocytes react in this antigen-specific manner to produce antibodies. These soluble proteins help fight infections by activating the complement cascade, enhancing phagocytosis, and neutralizing bacterial toxins. Cytotoxic T lymphocytes kill virus-infected cells in an antigen-specific manner. Helper T lymphocytes regulate many different types of immune responses. Nonspecific defenses often provide an early line of defense while antigen-specific responses provide later, stronger responses that exhibit immunological memory. Alterations in total or relative white blood cell numbers could indicate either an immunological deficiency or an adaptive response to infections. Hence, white blood cell counts potentially are important biomarkers for the effects of environmental contaminants

on specific or nonspecific immunity. The present study provided evidence that organochlorine contaminants may influence hematological variables, but these effects were complicated by variability in responses between ages and by inconsistency in which measure of contamination had the greatest influence.

Coplanar HAHS, especially TCDD and some PCBs, bind to an intracellular receptor known as the aryl hydrocarbon (Ah) receptor, thereby inducing the expression of detoxification enzymes, including cytochrome P4501A (CYP1A). The activities of two CYP1A-associated enzymes, EROD and aryl hydrocarbon hydroxylase (AHH), have been used as biomarkers for exposure to coplanar HAHs in colonial fish-eating waterbirds from the Great Lakes (Boersma *et al.* 1986, Hoffman *et al.* 1987; Fox 1993), British Columbia (Bellward *et al.* 1990, Sanderson *et al.* 1994), and the Netherlands and Belgium (Murk *et al.* 1992, Van den Berg *et al.* 1994). EROD activity is an integrated measure of exposure to a number of contaminants that act via the Ah-receptor. Contaminants other than HAHS, including polycyclic aromatic hydrocarbons, can induce EROD. In the present study, EROD activity was highest in both chicks and adults at Hamilton Harbour, Lake Ontario, a site where there is significant contamination with polycyclic aromatic hydrocarbons from two large steel mills. In addition to influencing EROD activity, polycyclic aromatic hydrocarbons also are immunosuppressive agents and carcinogens (Luster *et al.* 1987).

In the present study, a positive relationship between PCB contamination and EROD activity held only for PCB exposures up to 1 μg/g in livers of chicks (wet weight) and 10 μg/g in livers of adults. Histological examination of liver tissues in adults revealed no lesions suggestive of hepatotoxicity at higher exposures. Organochlorines are metabolized by CYP1A and could compete with the ethoxyresorufin substrate in the EROD assay. However, analyses of a subset of the liver tissues from adult gulls in this study showed

that EROD activity was highly correlated with CYP1A protein as determined by immunoblot techniques (S. Kennedy, pers. commun.). Hence, low EROD activity was the result of low amounts of enzymatic protein and not inhibition of enzyme activity by alternate substrates. An important question raised by this study is what factors might have downregulated EROD activity at many of the sites with high HAH contamination?

One factor might be a genetic difference in Ah receptor expression and (or) activity. Polymorphism at the *Ah* gene locus makes some strains of laboratory animals more susceptible than others to Ah-receptor-active chemicals, including immunotoxic effects (Silkworth and Grabstein 1982, Silkworth *et al.* 1984, Nagarkatti *et al.* 1984, Kerkvliet *et al.* 1990a,b). It is possible that at highly contaminated Great Lakes sites, Ah-receptor-mediated toxicity has selected for herring gulls that have low Ah receptor activity, leading to lower EROD activity than expected based solely on contamination. Activity of the Ah receptor and exposure to HAHs *both* are important for determining Ah-receptor-mediated effects, including immunotoxicity and EROD activity.

In this study, white blood cell counts showed relationships to organochlorine contaminants more frequently in adults than in chicks (Tables 2.3 and 2.4). In chicks, the only significant relationship was for an increased heterophil to lymphocyte ratio at higher concentrations of HG-TEQs. One site, the Lake Winnipeg reference site, did not fit this relationship. Gulls from this low contamination site had high heterophil to lymphocyte ratios. The high ratios may have been an artifact of poor quality blood smears, which caused clumping of heterophils. This outlier site was not eliminated because there was a strong association even when it was included. This relationship suggests that high HAH contamination alters white blood cell subpopulations in favor of heterophils over lymphocytes in chicks. Although these data might suggest a deficiency in producing

lymphocytes at high HAH exposures, no effects were seen on total white blood cell counts in chicks. However, total counts were conducted in chicks at only three of the eleven sites. In adults, the heterophil to lymphocyte ratio increased with PCB contamination, similar to the association with HG-TEQs in chicks. On the other hand, in adults DDE tended to increase the number of total white blood cells and total heterophils. Because the heterophil to lymphocyte ratio did not show a significant trend with DDE contamination, the increase in total heterophils probably reflected the increased total white blood cell counts without a differential shift toward heterophils.

In contrast to these measures of organochlorine contamination, EROD exhibited inverse relationships with total white blood cells, total heterophils, and the heterophil to lymphocyte ratio in adult gulls. The strong downward trend in total white blood cells in association with increasing EROD was accompanied by a strong downward trend in total heterophils and a weaker downward trend in total lymphocytes (Table 2.4). Hence, as EROD increased, total white blood cell counts decreased, and there was a shift towards fewer heterophils relative to lymphocytes. EROD and other inducible liver enzymes metabolize steroid hormones, including adrenal corticosteroids. Contaminant-induced EROD activity has been associated with lower circulating concentrations of adrenal corticosteroids in fish that have been stressed by capture (Hontela *et al.* 1992, 1995). Low concentrations of corticosteroids decrease the heterophil to lymphocyte ratio (birds) or neutrophil to lymphocyte ratio (mammals; Gross and Siegel 1980, 1981). Hence, contaminant-induced changes in the activity of liver enzymes might be expected to alter relative white blood cell counts and hence immunological function. The apparent contradiction between the opposite effects of organochlorine residues and EROD activity on hematological variables might be explained by several factors. Only three of seven sites at

which total white blood cell counts were conducted fell on the portion of the exposure-response curve (less than 10 μg/g PCBs) where there was a good correlation between PCB concentrations and EROD activity. Hence, for four of the seven sites, EROD was not a clear index of HAH contamination, so it is difficult to draw conclusions about the effects of individual HAHs on hematological variables from the EROD data. Other contaminants or other factors could have acted in conjunction with PCBs to influence EROD activity. Even though the relationship between organochlorine contamination and EROD activity was not clear in this field study as compared to well-controlled laboratory studies, it does seem apparent that EROD activity influences hematological variables and probably immune function. Since PCBs and dioxins, among other factors, do influence EROD activity, the association between hematological variables and EROD activity suggests that organochlorine contaminants or associated Ah-receptor-mediated events can influence hematological variables. The alterations in differential white blood cell numbers associated with high EROD activity strongly suggests that the complex mixtures of contaminants in the Great Lakes exert toxic effects on immune systems of herring gulls.

Of the other biochemical biomarkers that often are affected by HAH exposure (vitamin A storage, plasma thyroxine, and hepatic concentration of highly carboxylated porphyrins), only the highly carboxylated porphyrins showed any correlations with hematological parameters. HAHs inhibit the enzyme uroporphyrinogen decarboxylase in the pathway of heme synthesis and cause the buildup of highly carboxylated porphyrins in the liver (Fox *et al.* 1988). No correlations were observed between carboxylated porphyrin concentrations and packed red blood cell volume. A causal mechanism by which highly carboxylated porphyrins could directly influence white blood cell counts is not readily apparent. The correlations among porphyrins and various white blood cell parameters

differed between ages. In adults, as porphyrins increased, there was a shift toward both a lower percentage and total number of heterophils even though the total leukocyte count did not increase. In chicks, as porphyrins increased, there was an increase in total leukocyte and total heterophil counts even though the heterophil to lymphocyte ratio did not change. Since the balance of heterophils to lymphocytes did not change significantly, the increase in total heterophils was probably the result of the increased total white blood cell count. Since heterophils generally made up a larger percentage of the total leukocyte counts than did lymphocytes, the total heterophil count followed the total white blood cell count more closely than did the total lymphocyte count.

Of the few investigations that have attempted to study hematological variables in herring gulls, most have focused on red blood cell parameters (Averbeck 1991, 1992). The PCV values found in the present study for herring gull adults and chicks were similar to those reported in other studies (Averbeck 1991, 1992). Averbeck (1992) studied hematological variables in clinically normal and abnormal herring and black-backed gulls from one colony in Germany. The abnormal gulls were emaciated, injured, oiled, or suffering from extensive parasite infections. Nestling herring gulls usually have lower PCVs than adults, but this difference disappears at fledging (Averbeck 1992). The maturation of red blood cell system before fledging is not surprising considering the high demands for oxygen transport for flight. The present study found statistically significant but biologically small differences in PCVs between adult males (mean=43.5%) and adult females (mean=42.5%). Averbeck (1991) found no significant difference in PCVs between sexes in adult herring and great black-backed gulls (*Larus marinus*). Averbeck (1992) found that the mean PCV in abnormal gulls was 33% as compared to 41% in normal gulls. In the present study, the PCVs of only a few adults approached the abnormal

values reported by Averbeck. Chronic dietary exposure to 50-100 μg/g of technical formulations of PCBs (Arochlors 1242 and 1254) have been shown to decrease PCV in chickens (Iturri 1974). In the present study, there was moderate evidence that PCV decreased as DDE increased in adults and strong evidence that PCV increased with EROD in chicks.

Averbeck (1992) also studied the differences in white blood cell counts between clinically normal and abnormal gulls. Total white blood cell counts did not differ between normal and abnormal gulls. Averbeck (1992) found that normal herring gulls had a mean percent heterophils of 35.5% and a mean percent lymphocytes of 60.9%. In abnormal gulls, these values shifted to 70.1% for heterophils and 25.7% for lymphocytes, although high variability did not allow the statistical differentiation of these groups.

Leighton (1984) conducted differential white blood cell counts on four week old herring gull chicks after dosing with crude oil. Chicks in the control group had a mean percent heterophils of 42.4% and a mean percent lymphocytes of 52.0%. The mean heterophil to lymphocyte ratio in these control chicks was 0.83. There was a dose-dependent increase in the heterophil to lymphocyte ratio. In the high dose group, which received 20 ml of crude oil/ kg/ day, the mean heterophil to lymphocyte ratio increased to 2.72 (70.4% heterophils and 27.8% lymphocytes). The increases in the heterophil to lymphocyte ratio in sick (Averbeck 1992) and oil-dosed (Leighton 1984) gulls would be expected following an adrenal stress response (Gross and Siegel 1980, 1981). Indeed, Leighton (1984) reported other pathological signs of adrenal stress associated with crude oil ingestion, including lipid depletion in the adrenal glands and thymic and bursal atrophy.

The total white blood cell counts in the present study were similar to those found by Averbeck (1992) at a single colony, although some sites in the present study had total

counts that were up to 30% lower. In the present study, there was much variation in the heterophil to lymphocyte ratio among sites, suggesting intercolony differences in factors such as environmental contaminants, weather, nutrition, disease, and disturbance that could differentially influence white blood cell populations. The range in heterophil to lymphocyte ratios found in the present study was similar to the range between normal and sick or oil-dosed gulls in other investigations (Averbeck 1992, Leighton 1984).

This study showed that hematological variables are useful biomarkers for assessing the health of wild herring gulls. Blood cell counts in adults showed greater variation among sites than in chicks. Several white blood cell variables showed strong relationships to various measures of organochlorine contamination, suggesting that such contamination may in fact alter immunological defenses against infectious agents. As in the National Toxicology Program's procedures for screening immunotoxic chemicals in laboratory animals (Luster *et al.* 1988b, 1994), white blood cell counts can be used in conjunction with more sophisticated tests of immune function to assess the immunotoxic risk presented by pollutants in wild animals. Additional field and laboratory research should be performed to investigate how factors such as multiple contaminants, stress, nutrition and infectious agents interact to influence hematological parameters and liver EROD activity.

Table 2.1. Organochlorine contaminants in pooled samples of livers from herring gull chicks from the Great Lakes, Lake Winnipeg, and the Atlantic coast during 1991 and 1992.

Colony	Lake, River, or Ocean	# of Liver Samples in Pool	Concentration (wet weight)						
			ΣPCBs (μg/g)	HG- TEQs (pg/g)[1]	p,p´-DDE (μg/g)	Dieldrin (μg/g)	Mirex (μg/g)	HCB (μg/g)	Heptachlor Epoxide (μg/g)
Kent Island	Atlantic O.	10	0.12	28.21	0.03	0.005	0.0001	0.0024	0.0008
Pony Island	L. Winnipeg	10	0.13	8.52	0.06	0.002	0.001	0.0030	0.0026
Double Island	L. Huron	10	0.24	15.84	0.13	0.016	0.002	0.0024	0.0071
Jackfish Bay	L. Superior	8	0.27	11.04	0.05	0.007	0.002	0.0015	0.0029
Chantry Island	L. Huron	10	0.67	24.18	0.14	0.021	0.020	0.0041	0.0204
Stracken Island	St. Lawrence R.	10	0.69	20.92	0.15	0.012	0.025	0.0032	0.0034
Hamilton Harbour	L. Ontario	7	0.79	63.50	0.40	0.052	0.040	0.0048	0.0037
Monroe	L. Erie	9	0.86	21.32	0.16	0.035	0.001	0.0027	0.0060
Scotch Bonnet Island	L. Ontario	8	0.87	68.24	0.56	0.020	0.099	0.0102	0.0074
Saginaw Bay	L. Huron	10	1.19	88.52	0.51	0.049	0.006	0.0070	0.0201
Upper Green Bay	L .Michigan	10	5.05	225.27	2.41	0.243	0.028	0.0178	0.0762

[1] Calculated toxic equivalents relative to TCDD based on experiments by Kennedy *et al.* 1994 using *in vitro* assays for EROD induction in primary hepatocyte cultures derived from herring gull embryos.

Table 2.2. Organochlorine contaminants in pooled samples of livers from herring gull adults from the Great Lakes, Lake Winnipeg, and the Atlantic coast during 1991 and 1993.

Colony	Lake, River, or Ocean	# of Liver Samples in Pool	Concentration (wet weight)						
			PCBs (μg/g)	HG- TEQs (pg/g)[1]	p,p′-DDE (μg/g)	Dieldrin (μg/g)	Mirex (μg/g)	HCB (μg/g)	Heptachlor Epoxide (μg/g)
Kent Island	Atlantic O.	20	1.81	42	0.59	0.017	0.012	0.0129	0.0051
Jackfish Bay	L. Superior	10	4.97	677	1.62	0.091	0.033	0.0186	0.0434
Granite Island	L. Superior	10	4.99	216	1.98	0.133	0.042	0.0194	0.0717
Lower Green Bay	L. Michigan	10	5.40	416	0.91	0.146	0.018	0.0006	0.0721
Chantry Island	L. Huron	15	5.63	196	1.73	0.117	0.102	0.024	0.0475
Hamilton Harbour	L. Ontario	10	9.80	242	3.21	0.075	0.607	0.0258	0.0159
Pony Island	L. Winnipeg	20	11.74	242	6.43	0.147	0.125	0.0351	0.0687
Upper Green Bay	L. Michigan	10	12.15	396	7.18	0.573	0.238	0.0396	0.2003
Scotch Bonnet Island	L. Ontario	8	13.60	422	7.40	0.076	1.270	0.0455	0.0363
Middle Sister Island	L. Erie	8	14.25	224	1.46	0.067	0.032	0.0232	0.0573
Fighting Island	Detroit R.	10	15.57	169	1.50	0.069	0.034	0.0265	0.0174
Middle Island	L. Erie	20	21.56	2117	2.21	0.101	0.059	0.0254	0.0383
Saginaw Bay	L. Huron	9	23.84	634	6.51	0.123	0.116	0.0370	0.0385

[1] Calculated toxic equivalents relative to TCDD based on experiments by Kennedy *et al.* 1994 using *in vitro* assays for EROD induction in primary hepatocyte cultures derived from herring gull embryos.

Table 2.3. Effects of contaminants on general immunological and hematological variables in herring gull chicks from the Great Lakes, Lake Winnipeg, and the Atlantic coast during 1991 and 1992. Organochlorine contaminants were measured in pooled liver samples.

Dependent Variable	Independent Variable	Predicted Trend[1]	Actual Trend	Test for Ordered Alternatives	
				Jonckheere Statistic	P-Value
Total WBCs	PCBs HG-TEQs DDE EROD	+/-	-	35	0.37
Total Heterophils	PCBs HG-TEQs DDE EROD	+/-	-	25	0.56
Total Lymphocytes	PCBs HG-TEQs DDE EROD	+/-	-	23	0.59
Heterophil/ Lymphocyte Ratio (Log_{10})	PCBs	+/-	+	267	0.35
	HG-TEQs	+/-	+	**981**	**0.0016**
	DDE	+/-	+	371	0.21
	EROD	+/-	-	473	0.11
PCV	PCBs	+/-	-	-217	0.63
	HG-TEQs	+/-	-	-245	0.67
	DDE	+/-	-	-175	0.52
	EROD	+/-	+	**559**	**0.0056**

[1] "+/-" means that the direction of the trend could not be predicted *a priori*.

Table 2.4. Effects of contaminants on general immunological and hematological variables in herring gull adults from the Great Lakes, Lake Winnipeg, and the Atlantic coast during 1991 and 1993. Organochlorine contaminants were measured in pooled liver samples.

Dependent Variable	Independent Variable	Predicted Trend[1]	Actual Trend	Test for Ordered Alternatives	
				Jonckheere Statistic	P-Value
Total WBCs	PCBs	+/-	-	80	0.55
	HG-TEQs	+/-	+	50	0.64
	DDE	+/-	+	**846**	**0.0004**
	EROD	+/-	-	**598**	**0.0016**
Total Heterophils	PCBs	+/-	-	366	0.046
	HG-TEQs	+/-	-	84	0.67
	DDE	+/-	+	**600**	**0.0016**
	EROD	+/-	-	**848**	**0.0004**
Total Lymphocytes	PCBs	+/-	+	**570**	**0.004**
	HG-TEQs	+/-	+	338	0.074
	DDE	+/-	+	214	0.22
	EROD	+/-	-	320	0.086
Heterophil/ Lymphocyte Ratio (Log_{10})	PCBs	+/-	-	823	0.17
	HG-TEQs	+/-	-	447	0.46
	DDE	+/-	+	17	0.94
	EROD	+/-	-	**3455**	**0.0004**
PCV	PCBs	+/-	-	-421	0.19
	HG-TEQs	+/-	-	**205**	**0.022**
	DDE	+/-	-	**-5**	**0.044**
	EROD	+/-	+	-1293	0.96

[1] "+/-" means that the direction of the trend could not be predicted *a priori*.

Table 2.5. Means (± one standard error) of hematological variables in herring gull chicks from the Great Lakes, Lake Winnipeg, and the Atlantic coast during 1991 and 1992. Numbers in parentheses indicate sample sizes. Study sites are listed in increasing order of liver PCB contamination.

Colony	WBC Counts (% or cells × $10^3/\mu l$)						Heterophil/ Lymphocyte Ratio	PCV (%)
	Total WBCs[1]	Heterophils	Lymphocytes	Monocytes	Basophils	Eosinophils		
Kent Island		58.9±4.6 (10)	35.8±4.2	3.1±1.0	2.0±0.3	0.1±0.1	2.00±0.36	31.7±0.8 (10)
	17.8±1.6 (9)	10.0±0.9	6.8±1.2	0.7±0.2	0.3±0.1	0.0±0.0		
Pony Island		51.4±7.6 (10)	40.5±6.9	6.0±1.1	2.2±0.5	0.0±0.0	2.11±0.67	27.2±1.0 (10)
Double Island		41.3±3.5 (10)	55.2±3.3	3.0±0.9	0.5±0.4	0.0±0.0	0.81±0.11	35.7±3.2 (10)
Jackfish Bay		30.4±2.6 (8)	63.9±3.0	3.4±0.8	2.2±0.4	0.0±0.0	0.50±0.06	29.4±1.2 (8)
Chantry Island		50.8±5.2 (10)	43.3±4.9	4.2±0.5	1.4±0.4	0.4±0.2	1.43±0.28	32.8±0.6 (10)
Stracken Island		34.9±4.3 (10)	62.2±4.3	2.6±0.3	0.2±0.1	0.0±0.0	0.63±0.12	33.1±0.7 (10)
Hamilton Harbour		51.6±5.5 (7)	40.8±5.4	4.0±0.7	3.6±0.8	0.0±0.0	1.44±0.24	32.6±1.3 (7)
Monroe								32.6±1.4 (9)
Scotch Bonnet Island		45.6±4.5 (7)	46.6±4.9	5.9±1.1	1.8±0.3	0.0±0.0	1.11±0.22	28.7±1.0 (7)
Saginaw Bay		54.2±4.8 (10)	36.3±3.9	7.4±1.4	1.0±0.8	1.0±0.5	1.71±0.25	30.1±1.1 (10)
	18.3±2.1 (9)	10.4±1.8	6.5±1.1	1.2±0.2	0.1±0.1	0.1±0.0		
Upper Green Bay		55.6±3.3 (10)	38.5±2.7	4.9±0.7	0.9±0.4	0.2±0.1	1.59±0.23	29.3±1.2 (10)
	17.4±4.3 (7)	10.7±3.4	5.7±0.9	0.8±0.2	0.1±0.0	0.0±0.0		

[1] Counts of total heterophils, lymphocytes, monocytes, basophils, and eosinophils (cells × $10^3/\mu l$) are next to total WBC counts in the second line for each site. Total white blood cell counts were conducted only at Kent Island, Saginaw Bay, and Upper Green Bay.

Table 2.6. Means (± one standard error) of hematological variables in herring gull adults from the Great Lakes, Lake Winnipeg, and the Atlantic coast during 1991 and 1993. Numbers in parentheses indicate sample sizes. Study sites are listed in increasing order of liver PCB contamination.

Colony	WBC Counts (% or cells x $10^3/\mu l$)						Heterophil/ Lymphocyte Ratio	PCV (%)
	Total WBCs[1]	Heterophils	Lymphocytes	Monocytes	Basophils	Eosinophils		
Kent Island		60.0±2.2 (20)	31.1±2.7	5.0±0.7	3.7±0.6	0.1±0.0	2.27±0.22	44.9±0.5 (20)
Jackfish Bay		58.1±5.0 (10)	35.3±4.8	3.0±1.0	2.5±0.7	1.1±0.6	2.64±0.87	42.0±1.2 (10)
	11.1±0.9 (10)	6.4±0.7	4.0±0.7	0.3±0.1	0.3±0.1	0.1±0.0		
Granite Island		61.4±4.4 (10)	28.6±3.2	6.6±1.8	3.3±0.6	0.05±0.05	2.94±0.91	41.8±0.7 (10)
	13.6±0.9 (10)	8.4±1.0	3.8±0.5	0.9±0.2	0.4±0.1	0.0±0.0		
Lower Green Bay		45.4±5.8 (10)	48.6±6.1	3.8±0.6	2.1±0.4	0.2±0.1	1.26±0.30	41.9±0.8
	10.6±0.7 (10)	4.9±0.7	5.1±0.7	0.4±0.1	0.2±0.0	0.0±0.0		
Chantry Island		61.1±3.2 (15)	29.9±3.4	5.5±0.7	3.3±0.7	0.3±0.1	2.93±0.64	44.5±0.8 (15)
Hamilton Harbour		41.8±3.2 (10)	52.2±3.9	4.6±1.1	1.3±0.5	0.05±0.05	0.89±0.14	44.6±0.6(10)
Pony Island		66.8±3.1 (19)	24.3±3.2	5.7±0.6	2.7±0.4	0.4±0.2	4.60±1.16	41.9±0.5 (20)
Upper Green Bay		66.4±4.9 (10)	26.2±4.9	4.8±0.9	2.4±0.6	0.3±0.2	4.48±1.5	44.0±0.9 (10)
	15.1±1.4 (10)	10.0±1.1	4.1±1.0	0.7±0.1	0.3±0.1	0.0±0.0		
Scotch Bonnet Island		47.6±5.6 (8)	42.9±5.5	6.1±1.3	3.2±0.5	0.2±0.1	1.48±0.44	41.5±1.0 (8)
	16.7±1.2 (8)	8.1±1.3	7.0±1.0	1.0±0.2	0.5±0.1	0.0±0.0		
Middle Sister Island								44.0±0.8 (7)
Fighting Island		46.8±3.3 (10)	46.4±3.0	3.8±0.7	2.4±0.4	0.6±0.3	1.09±0.14	42.4±0.7 (10)
	10.0±0.9 (10)	4.8±0.7	4.5±0.4	0.4±0.1	0.2±0.0	0.0±0.0		
Middle Island		64.8±3.4 (20)	27.0±3.6	4.4±0.8	3.3±0.6	0.5±.01	3.83±0.63	43.3±0.5 (20)
Saginaw Bay		35.4±4.5 (10)	58.0±4.2	4.0±0.5	2.7±0.4	0.0±0.0	0.72±0.17	42.2±0.6 (10)
	13.2±1.6 (10)	5.1±1.3	7.2±0.6	0.5±0.1	0.3±0.0	0.0±0.0		

[1] Counts of total heterophils, lymphocytes, monocytes, basophils, and eosinophils (cells x $10^3/\mu l$) are next to total WBC counts in the second line for each site. Total white blood cell counts were conducted at Jackfish Bay, Granite Island, Lower Green Bay, Upper Green Bay, Scotch Bonnet Island, Fighting Island, and Saginaw Bay.

Table 2.7. Pearson's correlation analysis exploring relationships among hematological biomarkers and other biomarkers in herring gull chicks and adults from the Great Lakes, Lake Winnipeg, and the Atlantic coast during 1991-93.

Relationship	Age	Variables		r	N	P
Among Hematological and Biochemical Variables	Adult	H/L Ratio[1]	Porphyrins	-0.31	152	<0.001
		Total Heterophils	Porphyrins	-0.32	68	0.009
		Total WBCs	Porphyrins	-0.17	68	0.18
	Chick	H/L Ratio	Porphyrins	0.20	92	0.058
		Total Lymphocytes	Porphyrins	0.70	25	<0.001
		Total WBCs	Porphyrins	0.70	25	<0.001
Hematological Variables and Body Size	Chick	H/L Ratio	Body Mass	-0.29	92	0.006
		H/L Ratio	Wing Chord	0.40	92	<0.001
		PCV	Body Mass	0.31	101	<0.001
Hematological Variables and Organ Masses	Chick	H/L Ratio	Thyroid Mass	-0.32	91	0.0002

[1] Heterophil/Lymphocyte Ratio

A.

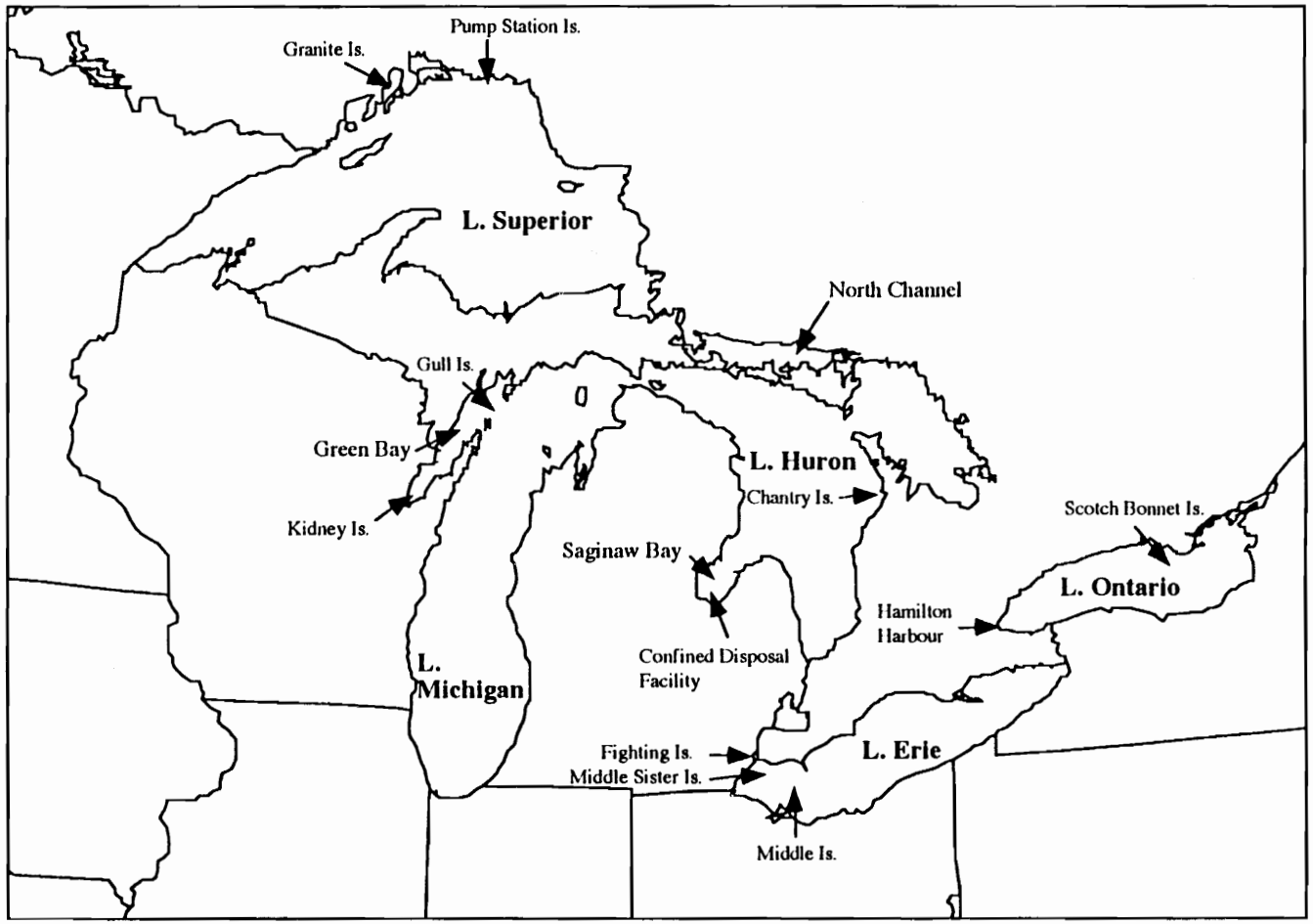

Fig. 2.1. Great Lakes sampling sites for immunological and hematological biomarkers in herring gull adults (A.) and chicks (B.). Adults and chicks were also sampled on Pony Island in northern Lake Winnipeg, Manitoba, and on Kent Island in the Bay of Fundy off Canada's Atlantic coast. Birds were sampled during 1991 with the following exceptions. Chicks were collected from Double Island, Stracken Island, and western Lake Erie during 1992, and adults were collected from Middle Sister Island during 1993.

B.

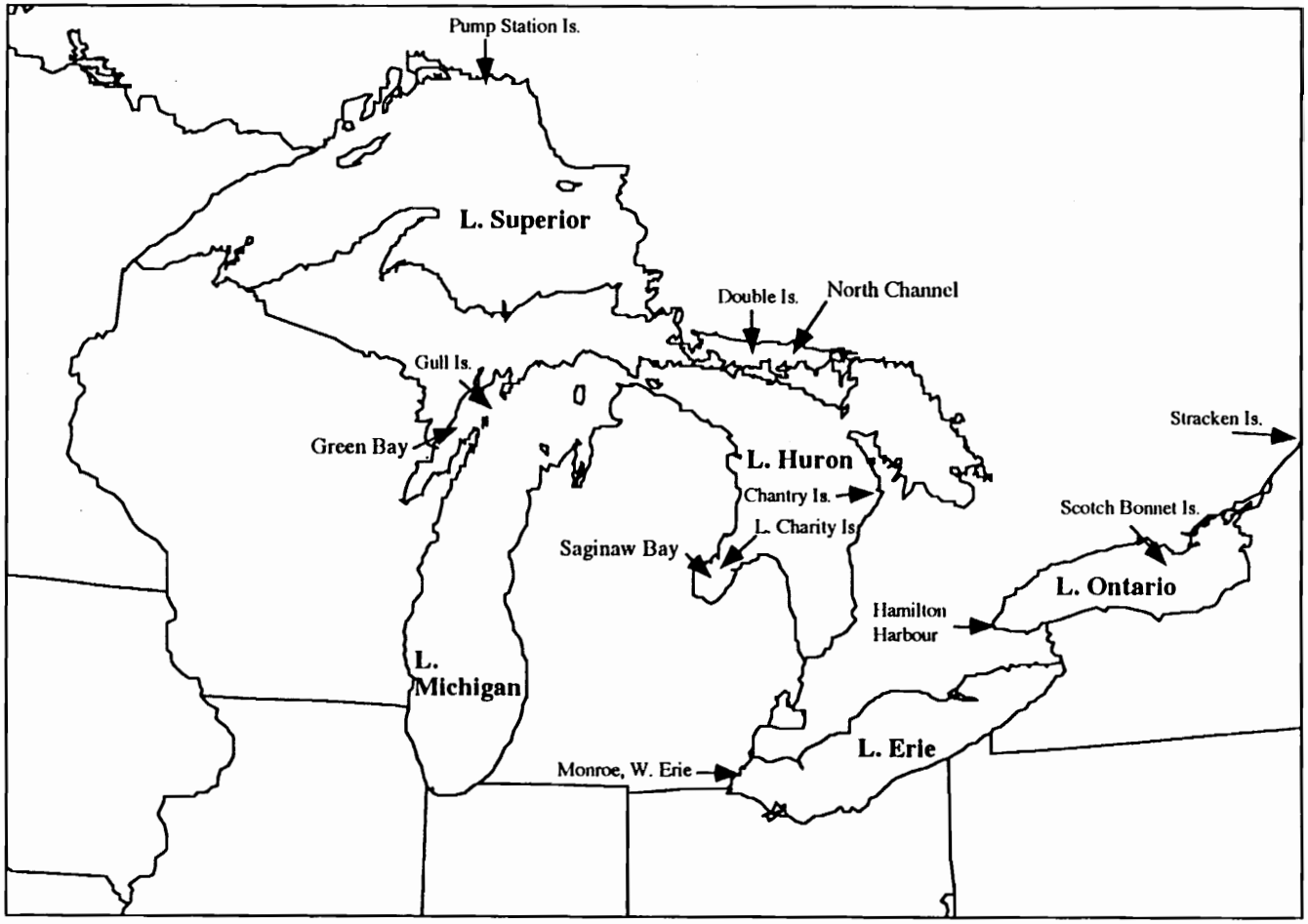

Fig. 2.1. (continued).

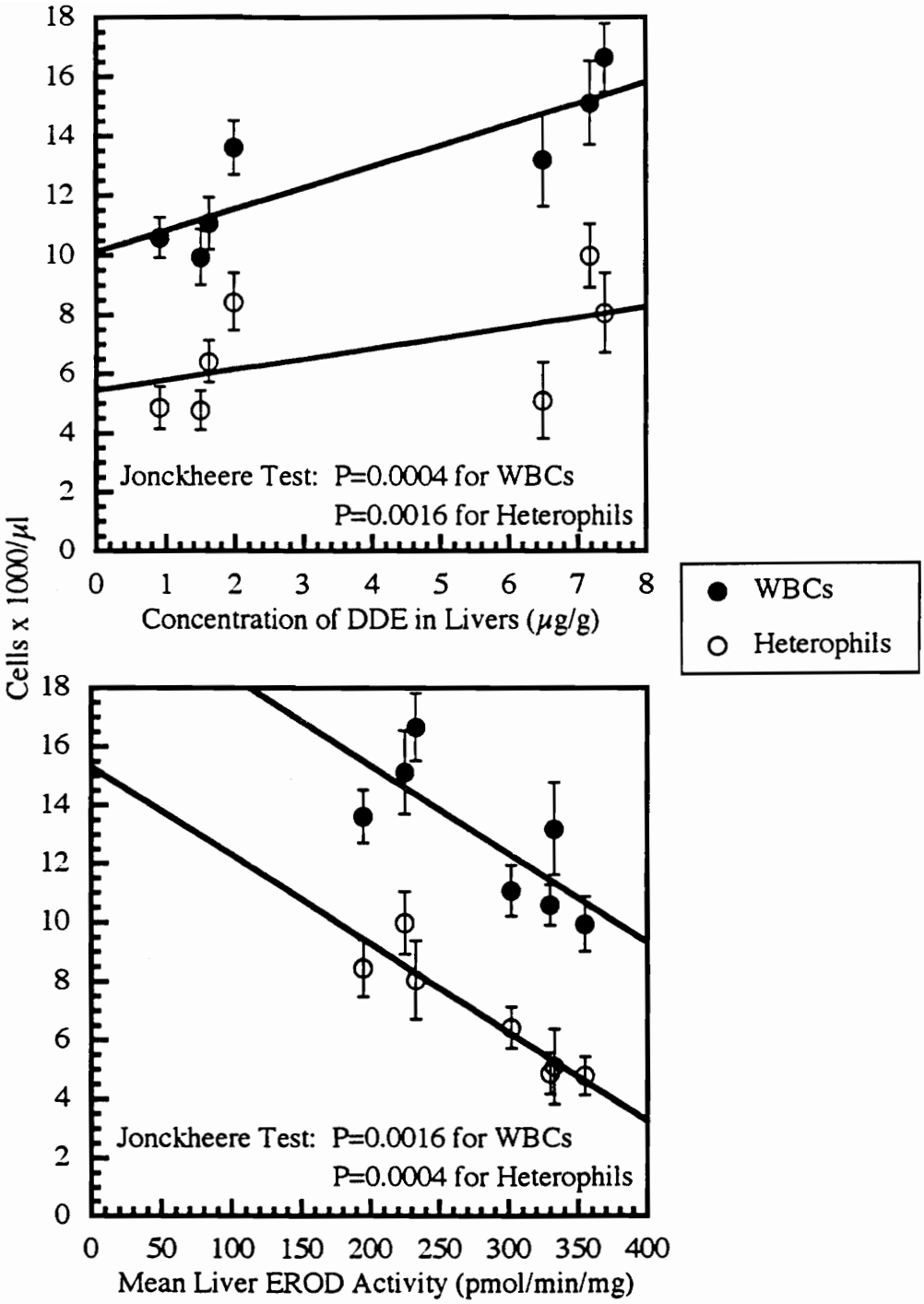

Fig. 2.2 Relationships between environmental contamination and total white blood cell and total heterophil numbers herring gull adults from the Great Lakes during 1991 and 1993. Error bars indicate one standard error of the mean.

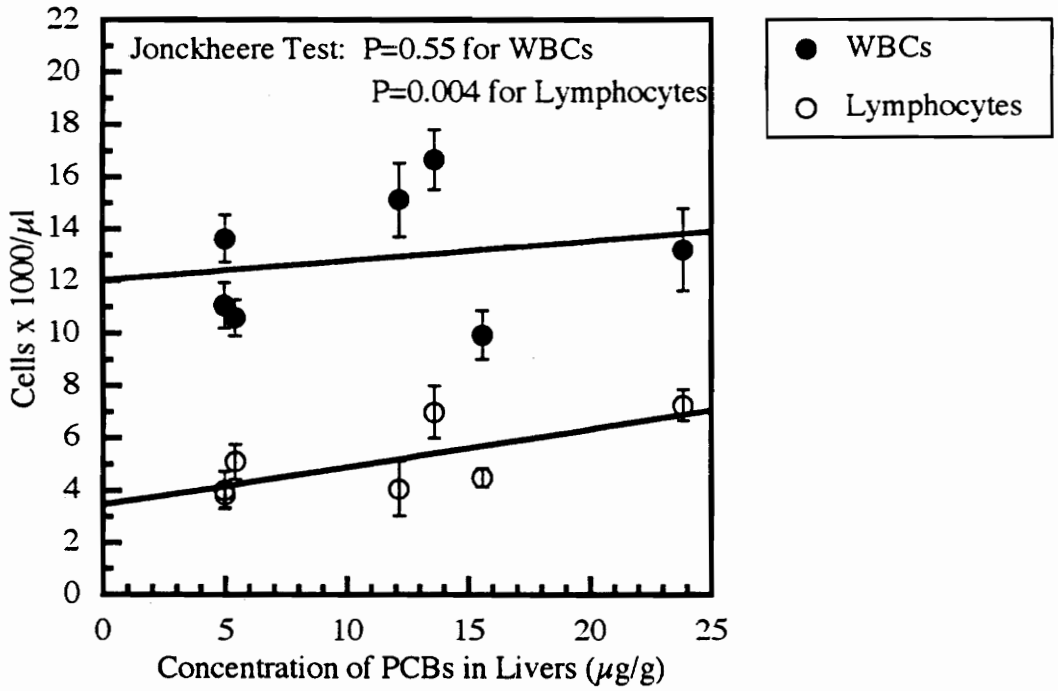

Fig. 2.3. Relationships between PCB contamination and total white blood cell and total lymphocyte numbers herring gull adults from the Great Lakes during 1991 and 1993. Error bars indicate one standard error of the mean.

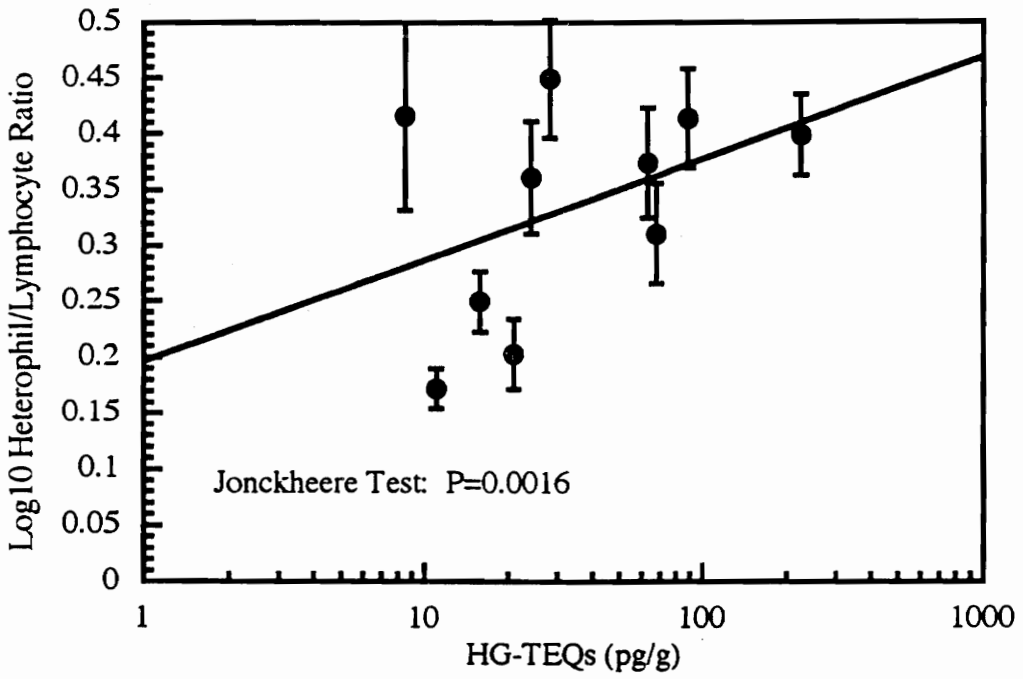

Fig. 2.4. Relationships between HG-TEQs and the heterophil/lymphocyte ratio in herring gull chicks from the Great Lakes, Lake Winnipeg, and the Atlantic coast during 1991 and 1992. Error bars indicate one standard error of the mean.

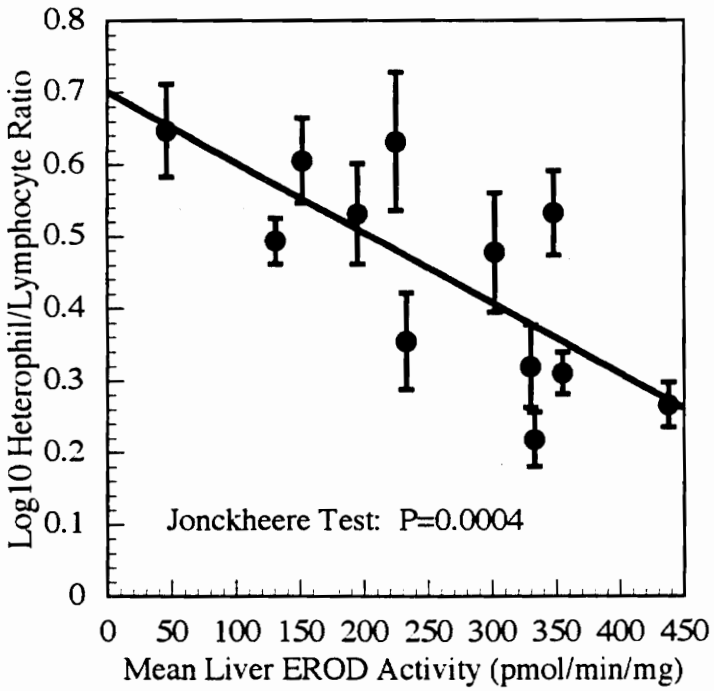

Fig. 2.5. Relationships between liver EROD activity and the heterophil/lymphocyte ratio in herring gull adults from the Great Lakes, Lake Winnipeg, and the Atlantic coast during 1991 and 1993. Error bars indicate one standard error of the mean.

Chapter 3. Immune and Endocrine Organ Masses as Biomarkers for Environmental Contaminants in Herring Gulls (*Larus argentatus*) of the Great Lakes

Summary

This study explored the feasibility of using immune and endocrine organ masses as immunological biomarkers for assessing the health of herring gulls, especially in relation to the effects of persistent environmental contaminants. If important structural components for the development of immunological cells are disrupted, it is likely that physiological functions also will be compromised. There was strong evidence that thymus mass decreased as the activity of ethoxyresorufin-O-deethylase (EROD) in the liver increased, although thymus mass did not show any significant relationships to concentrations of individual organochlorine contaminants in liver samples. Bursa and spleen masses did not show any associations with organochlorine contaminants or liver EROD activity. However, the role of contaminants in growth of the bursa was complicated by the presence of bursitis caused by fluke infestations (*Cotylurus communis*). Thyroid mass increased with polychlorinated biphenyl (PCB) contamination and liver EROD activity in herring gull adults. In chicks, thyroid mass increased with EROD but decreased with HG-TEQs (dioxin toxicity equivalents as calculated from herring gull-specific toxicity equivalency factors). In adults, there was very strong evidence that thyroid mass increased with liver PCBs , HG-TEQs, and EROD activity. Although the role of organochlorines in influencing immune organ mass was complicated by factors such as multiple contaminants,

infections, and nutrition, the association between thymic atrophy and increasing EROD suggests a significant Ah-receptor mediated effect that could have severe consequences on immune function.

Introduction

Biomarkers are biochemical, physiological, or histological changes that measure effects of, or exposure to, toxic chemicals. Some biomarkers such as organ masses and histology may indicate alterations in biological structure that strongly suggest alterations in biological function. Other biomarkers such as enzyme activities and hormone levels more directly measure function. Many biochemical and developmental biomarkers in colonial fish-eating birds have been used effectively to monitor exposure to and the effects of organochlorine contaminants in the Great Lakes during the past 25 years (Fox 1993), but there have been no investigations on the effects of pollutants on the immune systems of these birds. This study examined the use of immune and endocrine organ masses as structural biomarkers for immunotoxicity in Great Lakes birds.

Today, much interest in the immunotoxic effects of environmental pollutants has focused on halogenated aromatic hydrocarbons (HAHs), which include polychlorinated biphenyls (PCBs), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and 2,3,7,8-tetrachlorodibenzo-*p*-furan (TCDF). These chemicals cause a variety of effects in laboratory animals, but one of the most-characteristic effects is immunosuppression (Silkworth and Vecchi 1985, Thomas and Faith 1985, Luster *et al.* 1987, and Vos and Luster 1989). In laboratory mammals and birds, HAH-induced immunosuppression can lead to increased susceptibility to challenges with bacteria (Thomas and Hinsdill 1978, Vos

et *al.* 1978, Hinsdill *et al.* 1980), viruses (Friend and Trainer 1970, Imanishi *et al.* 1980, Vos and Luster 1989, House *et al.* 1990), and protozoan parasites (Vos and Luster 1989). At low doses in developing animals, HAHs often suppress regulatory and effector functions of T lymphocytes (Vos and Van Driel-Grootenhuis 1972; Sharma *et al.* 1978; Hinsdill *et al.* 1980; Thomas and Hinsdill 1980; Clark *et al.* 1981, 1983; Takagi *et al.* 1987; Neubert *et al.* 1991; Tomar *et al.* 1991). Such effects are especially pronounced and long-lasting in developing and growing animals (Takagi *et al.* 1987, Holladay and Luster 1994). At higher doses, HAHs often suppress antibody production by B lymphocytes (Sharma *et al.* 1978; Thomas and Hinsdill 1978; Hinsdill *et al.* 1980; Clark *et al.* 1981, 1983; Silkworth and Grabstein 1982; Silkworth *et al.* 1984; Luster *et al.* 1988a; House *et al.* 1990; Kerkvliet *et al.* 1990b).

While these immunotoxic effects of HAHs often have been detected using sophisticated tests of immune function, they also have been revealed by gross and microscopic structural changes in primary lymphoid organs, which are important sites for lymphocyte development. In birds, lymphocyte precursors migrate from hematopoietic tissues into the bursa of Fabricius and develop into B lymphocytes. After leaving the bursa, B lymphocytes become antibody-producing or memory cells if they are stimulated by the appropriate antigen. B lymphocytes develop in the bone marrow or Peyer's patches (gut-associated lymphoid tissue) in mammals. T lymphocytes develop in the thymus gland. After leaving the thymus, T lymphocytes play important regulatory and cytotoxic roles in immunological responses. In laboratory rodents exposed to sublethal doses of TCDD and coplanar PCBs, thymic atrophy is one of the most characteristic effects (Vos and Van Driel-Grootenhuis 1972, Silkworth and Grabstein 1982, Vos and Luster 1989). This atrophy is characterized by a depletion of lymphocytes in the thymic cortex, which

may be a reflection of a reduction in the ability of the thymic cortex to support the maturation of T cells (Greenlee *et al.* 1985). Harris *et al.* (1976) observed decreased spleen and bursa of Fabricius masses in the offspring of chicken hens (*Gallus domesticus*) fed PCBs. Thymus masses were not reported. Coplanar PCBs and TCDD have produced dose-dependent decreases in the number of viable lymphocytes in the thymus and bursa of Fabricius of chicken embryos that developed in eggs that had been injected with HAHs (Nikolaidis *et al.* 1988a,b, Andersson *et al.* 1991). The ratios of thymus mass to body mass and spleen mass to body mass have been included as Tier I tests for screening chemicals for immunotoxic potential in the National Toxicology Program (Luster *et al.* 1988b, 1994). The measurement of bursal mass in birds is a logical extension. Although these simple variables often are less sensitive than direct tests of immune function, a significant alteration in the size or structure of lymphoid organs is usually associated with abnormal immune function.

Because of the close connection between the immune and endocrine systems, endocrine organ mass and associated hormones also might influence immune function. Organochlorine-associated goiter has been demonstrated in Great Lakes herring gulls (Moccia *et al.* 1986, Fox 1993). Adrenal corticosteroids are potent immunosuppressive and anti-inflammatory hormones. Retinoid, or vitamin A, is important for proper development, immune function, and vision. Environmental contaminants such as HAHs can disrupt vitamin A homeostasis (Zile 1992), and such effects have been observed in fish-eating birds of the Great Lakes (Spear *et al.* 1985, 1990; Fox 1993).

Although many physiological, pathological, nutritional, and ecological factors can affect immune function, HAHs consistently influence these parameters. Numerous studies have documented significant reproductive, developmental and behavioral impacts of these

chemicals on fish-eating colonial waterbirds in the Great Lakes basin (Peakall *et al.* 1980; Mineau *et al.* 1984; Fox and Weseloh 1987; Peakall and Fox 1987; Gilbertson 1988, 1989; Peakall 1988; Colborn *et al.* 1990; Fox *et al.* 1991; Gilbertson *et al.* 1991; Government of Canada 1991; Fox 1993). At sites that are highly contaminated with PCBs, colonial waterbirds have experienced a number of severe developmental effects, which include high embryonic mortality, subcutaneous, pericardial, and peritoneal edema, congenital deformities, growth retardation, hepatomegaly, liver necrosis, and liver porphyria (Gilbertson 1989). Because the immune system is often more sensitive than other physiological systems to environmental contaminants, especially in young animals, Great Lakes colonial waterbirds are likely also experiencing immunotoxic effects. At highly contaminated sites in the Great Lakes, double-crested cormorants (*Phalacrocorax auritus*) have an abnormally high incidence of eye infections (Ecological Research Services 1991). Laboratory experiments suggest that measurements of immune and endocrine organ mass might be good biomarkers for effects of HAHs in wild animals. These simple measurements, which are easy to obtain during necropsy, might substitute for more sophisticated tests of immune function that are difficult to conduct under field conditions.

This investigation was part of a larger study that sought to determine the effects of environmental contaminants on immunocompetence in herring gulls (*Larus argentatus*) in the Great Lakes. It examined the usefulness of structural biomarkers such as immune and endocrine organ masses for monitoring contaminants, and it compared these structural biomarkers to several common biochemical biomarkers related to immune function and (or) contaminant exposure.

Materials and Methods

Sampling Design

Study sites were chosen across a wide range of organochlorine contamination (Table 2.1). Adult herring gulls were sampled from eleven sites within the Great Lakes (Fig. 2.1.A): 1) an officially unnamed island (Pump Station Island) near Jackfish Bay, northern Lake Superior; 2) Granite Island in Black Bay, north shore of Lake Superior; 3) Gull Island in upper Green Bay, Lake Michigan; 4) Kidney Island in lower Green Bay, Lake Michigan; 5) Confined Disposal Facility in Saginaw Bay, Lake Huron; 6) Chantry Island, southeastern Lake Huron; 7) Fighting Island, in the Detroit River; 8) Middle Island, western Lake Erie; 9) Middle Sister Island, western Lake Erie; 10) Hamilton Harbour, western Lake Ontario; and 11) Scotch Bonnet Island, eastern Lake Ontario. Twenty-eight-day-old herring gull chicks were collected from nine Great Lakes sites (Fig. 2.1.B): 1) an officially unnamed island (Pump Station Island) near Jackfish Bay, northern Lake Superior; 2) Gull Island in Upper Green Bay, Lake Michigan; 3) Double Island in the North Channel of Lake Huron; 4) Little Charity Island in Saginaw Bay, Lake Huron; 5) Chantry Island, southeastern Lake Huron; 6) Monroe, western shore of Lake Erie; 7) Hamilton Harbour, western shore of Lake Ontario; 8) Scotch Bonnet Island, eastern Lake Ontario; and 9) Stracken Island, St. Lawrence River. For both ages of herring gulls, two reference sites outside the Great Lakes were sampled: Kent Island in the Bay of Fundy off Canada's Atlantic coast; and Pony Island, in the northern end of Lake Winnipeg, Manitoba. Total white blood cell counts were conducted at seven sites for adults and three sites for chicks. Differential white blood cell counts were not made on adults at Middle Sister Island and chicks from Monroe, Lake Erie. Adult gulls were sampled during 1993 at Middle

Sister Island. Chicks were collected during 1992 at Double Island, Stracken Island, and Monroe. All other collections were made during the 1991 breeding season.

Adult herring gulls were captured by drop-trapping over nests during mid-incubation. Prior to trapping, eggs were floated to determine the buildup of air inside the egg and hence the approximate stage of incubation. Herring gull chicks were randomly selected and captured at the approximately 28 days of age. Approximately ten birds of each age were collected at each of the study sites listed above. For several sites, 15-20 birds were collected to determine whether sample sizes larger than ten provide significantly greater sensitivity for biomonitoring. An effort was made to collect equal numbers of adult males and females from each site.

Hematology

A blood sample (approximately 11 ml) was drawn from the brachial vein using a 22 gauge needle and Vacutainer® tubes (Beckton Dickinson, Rutherford, NJ). Seven ml of blood were drawn into a tube containing dry sodium heparin and 4 ml into a tube containing dry potassium EDTA. Two blood smears were made quickly from the blood collected in EDTA. The heparinized blood was centrifuged immediately at 2575×g for 5 minutes, and the plasma was stored on liquid nitrogen for vitamin A and thyroxine analysis. The PCV was measured by centrifuging microhematocrit tubes filled with blood collected in EDTA at 5125×g for five minutes in a clinical centrifuge equipped with a hematocrit rotor.

The blood collected in EDTA was kept on ice until a total white blood cell count was completed, usually on the same day as collection. Dilutions were made with standard 101-1 erythrocyte pipettes in modified Natt and Herrick's diluent following the procedures

of Gross (1984). Leukocytes were counted under a Neubauer hemacytometer at 400× magnification.

Blood smears were air-dried and fixed in methanol to preserve the cells until staining. Smears were stained with Wright stain (Accustain™, Sigma, St. Louis, MO) using 100% stain for 30 seconds and then diluting with an equal volume of distilled water for 90 seconds. Smears were rinsed with distilled water and allowed to air dry. Two hundred white blood cells were counted and classified using oil immersion microscopy at 1000× magnification. The total number of each type of white blood cell was determined by multiplying its percentage from the differential count by the total leukocyte count.

Dissection and Tissue Preservation

Before dissection, the body size of each gull was determined by measuring body mass, head length, bill depth, foot length, and, in chicks, wing chord (Fox *et al.* 1981). All chicks of appropriate size and all adults were sacrificed by decapitation using a guillotine and allowed to bleed out fully. The liver was quickly removed. The left lobe of the liver was later to be used for ethoxyresorufin-O-deethylase (EROD) analysis. This lobe was wrapped in high density polyethylene film, placed in 20 ml polyethylene scintillation vials, and preserved in liquid nitrogen within ten minutes after death. The concentrations of vitamin A, highly carboxylated porphyrin, and organochlorine contaminants were to be measured in the right lobe. Eight 1 gram samples of the right lobe of the liver were placed in 1 ml vials and preserved in liquid nitrogen for biomarker analysis. The remainder of the right lobe of the liver was placed in an acetone-hexane-rinsed glass jar and transported to the laboratory on wet ice before freezing at -20 °C prior to organochlorine analysis. The gonads, liver, adrenal glands, thyroid glands, and spleen of each bird were preserved in a

neutral buffered formalin solution (10%). The thymus gland and bursa of Fabricius in chicks also were preserved in this fashion. In the laboratory, the thyroids, adrenals, spleen, thymus, and bursa were trimmed from associated connective tissue, blotted, and weighed to the nearest 0.01 g. Standard paraffin sections of the spleen, thymus, bursa, and liver were stained with hematoxylin and eosin and examined by avian histopathologists using standard histopathological techniques.

Biochemical Biomarkers

Vitamin A compounds in the liver were extracted from 0.3-0.5 g liver samples and dehydrated by grinding with anhydrous sodium sulphate. Powder equivalent to 0.20 g of liver was combined with 8-20 ng of retinyl acetate in 20 µl of 2-propanol as an internal standard. Retinoids were extracted in 10 ml of dichloromethane:methanol (1:9) by repeated shaking and liberation of pressure for 5 minutes. The extract was centrifuged at 600 rpm and 10 °C for 10 minutes. An aliquot of the supernatant was filtered through a 0.20 or 0.45 µm filter disk. The final extract (20 µl) was analyzed by nonaqueous reverse-phase HPLC.

Plasma retinol was extracted from 100 µl of plasma after the addition of 75 ng of retinyl acetate in 15 µl of 2-propanol as an internal standard. Retinol-protein complexes were dissociated by vigorous shaking after the addition of 200 µl of acetonitrile. The retinol was extracted with successive 4 and 1 ml volumes of hexane. After shaking for 5 minutes, the organic and aqueous phases were separated by centrifugation at 1500 rpm for 5 minutes at 4 °C. The organic phase was dried under nitrogen, reconstituted with 500µl of methanol, and filtered through a 0.2 µm PVDF filter disk.

The liver and plasma extracts were analyzed for vitamin A compounds by reverse-phase HPLC using an ODS spheri-5 guard column (Brownlee) and a 15 cm long, 5 μm ODS Zorbax (DuPont) analytical column. For the liver extract, the methanol and dichloromethane were used to resolve the retinoids in less than 10 minutes. Methanol was used to separate the retinoids in the plasma extract in less than 6 minutes. Either fluorescence (ex: 336 nm; em: 480 nm) or UV-visible (326 nm) was used to detect the retinoids. The detection limits were 5 μg/L retinol in plasma, 0.4 μg/g retinol in liver, and 1.2 μg/g retinyl palmitate in liver.

The left lobe of each liver was homogenized, and microsomes were isolated following the procedure of Pyykko (1983). The catalytic activity of EROD in the liver was determined using the methods of Kennedy and Jones (1994) optimized for herring gull liver microsomes. Highly carboxylated porphyrins were measured in liver tissue following the procedure of Kennedy and James (1993). In plasma samples from adults, total thyroxine was measured using a solid phase enzyme assay (veterinary modification of the EZ Bead T4 Test, Immunotech Corp, Boston, MA).

Organochlorine Analyses

The 10-20 individual liver samples collected for each age and site were pooled for organochlorine analysis by the analytical services laboratory at the National Wildlife Research Centre of the Canadian Wildlife Service following the methods of Peakall *et al.* (1986). PCB residues are reported as the sum of the following 42 PCB congeners: IUPAC nos. 28, 31, 42, 44, 49, 52, 60, 64, 66, 70, 74, 87, 97, 99, 101, 105, 110, 118, 128, 129, 137, 138, 141, 146, 149, 151, 153, 158, 170, 171, 172, 174, 180, 182, 183, 185, 194, 195, 200, 201, 203, and 206.

Nonortho PCB congeners (IUPAC nos. 37,77,126, and 169) and all 2,3,7,8-substituted polychlorinated dibenzo-dioxin (PCDD) and polychlorinated dibenzo-furan (PCDF) congeners also were measured in pooled liver samples by the analytical services laboratory of the National Wildlife Research Centre of the Canadian Wildlife Service. Pooled liver samples were dried with anhydrous sodium sulphate and ground into a fine powder. An open chromatographic column wet-packed with multiple absorbents (anhydrous sodium sulphate, deactivated silica, sulfuric acid on silica, activated silica, and sodium hydroxide on silica) was used for the initial extraction and cleanup. The sample was spiked with a ^{13}C PCDD mixture (Cambridge Isotope Laboratories) and a ^{13}C PCB 77, 126, and 169 mixture (Wellington Isotope Laboratories) to determine the degree of analyte loss during sample workup. The column was eluted using 1:1 dicloromethane/hexane, and the eluted extract was concentrated by evaporation. A liquid chromatograph (FMS Systems Inc.) with a carbon column was used for further cleanup and trace enrichment. The carbon column was flushed with dichloromethane and backflushed with toluene. The eluent was concentrated by evaporation, and the solvent was changed to hexane. After concentration by evaporation, the extract was cleaned up and separated on a deactivated Florisil column wet packed in hexane. The column was first eluted with 1:20 dichloromethane/hexane for nonortho PCB analysis. The column was then eluted with dichloromethane to produce a second fraction that was cleaned up on a wet packed, activated basic alumina column (Fisher Scientific). The alumina column was eluted with 1:50 dichloromethane:hexane to produce a fraction containing residual PCBs and other organochlorines. The column was then eluted with 1:1 dichloromethane:hexane to produce a fraction for PCDD/PCDF analysis. A Hewlett-Packard 5971A GC/MSD was used to separate and quantify nonortho PCB, PCDD, and PCDF congeners. Detection

limits were 75 pg/g for nonortho PCB congeners and approximately 0.5-25 pg/g for PCDD and PCDF congeners.

Because different HAH congeners have different relative toxicities, the total biological activity of a complex mixture of congeners cannot be estimated by adding the concentrations of the individual congeners. Furthermore, the relative toxicities of different congeners vary from species to species. Kennedy *et al.* (1994) used *in vitro* induction of EROD activity in primary hepatocyte cultures from 26 day old herring gull embryos to compare the relative toxicities of TCDD, TCDF, and various PCBs. Based on the EC_{50} for EROD induction, different HAH congeners could be compared to TCDD, the most toxic congener. The following herring gull-specific toxicity equivalency factors (TEFs) were generated: TCDD = 1.0; TCDF = 0.9; PCB congener # 169 = 0.07, PCB congener #126 = 0.06; PCB congeners # 77, #105, #118=0. Multiplying the concentration of each congener by its TEF and then summing the products gave an estimate of the total dioxin-like toxicity of the mixture for herring gulls. This estimate was called HG-TEQs, which stands for herring gull-specific TCDD-equivalents.

Statistical Analyses

The primary goal of this investigation was to determine whether there was an association between the degree of organochlorine contamination and immunological variables in fish-eating birds of the Great Lakes. The purpose was to determine whether intercolony differences in immunological variables could be explained by exposure-response relationships. Statistical methods were needed to determine the probability that the spatial patterns in response variables were associated with different pollutants as opposed to being caused by random events. The purpose was not to show what percent of

variability in immunological responses could be explained statistically by particular chemicals. Because detailed organochlorine analyses are expensive and require large volumes of blood, contaminant concentrations were not measured in individual chicks. However, chemical analysis of pooled liver samples from each site allowed the sites to be ranked on the basis of concentrations of different contaminants.

Statistical methods were chosen to meet this primary goal of associating immunological variables with pollutants and to work within the constraints of pooled chemical analyses. Regression analysis was not appropriate because of its emphasis on explaining variability using linear models and its need for chemical analyses on individual birds. Although analysis of variance (ANOVA) could detect spatial differences in immune responses among sites, it was not appropriate because it could not be used to look for associations across gradients of contamination.

The Jonckheere test for ordered alternatives was used as the primary statistical method of testing specific hypotheses about contaminant-associated immunosuppression (Hollander and Wolfe 1973). This test fit the purpose and design of the study as well as the constraints on chemical analysis. The null hypothesis for this nonparametric measure of exposure-response states that there is no difference among the central tendencies from different sites (H_O: $\mu_1 = \mu_2 = \mu_3 = ... = \mu_n$). The alternative hypothesis is that there is a monotonic trend (not necessarily linear) in the data based on *a priori* information (H_A: $\mu_1 \geq \mu_2 \geq \mu_3 ... \mu_n$, where at least one of the inequalities is a strict inequality). Laboratory data were used to predict that contamination would cause a decrease in the mass of the thymus and bursa of Fabricius (Vos and Van Driel-Grootenhuis 1972, Silkworth and Grabstein 1982, Vos and Luster 1989, Harris *et al.* 1976) and an increase in the mass of the thyroid gland (Moccia *et al.* 1986, Fox 1993). The direction of the trends for the spleen

and adrenals could not be easily predicted from laboratory data, so a two-way test was performed by running the Jonckheere test in both directions and doubling the P-value for the most significant trend. Severe immunosuppression could have caused leukopenia and splenic atrophy, or recurring infections caused by immunosuppression could have caused leukocytosis and splenomegaly. The following variables were used to order the study sites: liver concentrations of total PCBs, DDE, and calculated HG-TEQs; and liver EROD activity. The Jonckheere test was performed using a custom-written FORTRAN program (B. Collins, Senior Statistician, Canadian Wildlife Service) using the algorithm provided by Gibbons (1971). This program determined probability values based on 5000 random permutations of the data set being analyzed. These probability values were very similar to those found by the large sample normal approximation for the Jonckheere test (Hollander and Wolfe 1973).

While the Jonckheere test was the primary statistical test, analysis of covariance (ANCOVA) with body mass as the covariate was used to for two purposes. First, ANCOVA was used to determine whether the response variable was influenced by sex or a sex × site interaction in adults. If there was no statistically significant sex or a sex × site interaction effect ($P<0.05$), then data were pooled across years for the Jonckheere test. Second, if the Jonckheere test revealed no significant relationships with contaminants, then ANCOVA was used to look for differences among sites. For ANCOVA analyses, bursal masses, spleen masses, and thyroid masses were transformed ($\log_{10}$) to satisfy assumptions of homogenous variances and normality (Zar 1984). Organ masses were converted to percent of body mass before analysis by the Jonckheere test or correlation but not before ANCOVA.

Relationships between the various biomarkers were detected using Pearson's correlation analysis. Statistically significant correlations ($P<0.05$) were reported only if the absolute value of the correlation coefficient (r) was greater than 0.3.

Results

Organochlorine Concentrations and EROD Activity

Organochlorine contaminants reached higher concentrations in herring gull adults than chicks (Tables 2.1 and 2.2). Minimum concentrations were 15.1× greater in adults for PCBs, 4.9× greater for HG-TEQs, and 19.7× greater for DDE. Maximum concentrations were 4.7× greater in adults for PCBs, 9.4× greater for HG-TEQs, and 2.7× greater for DDE. Although concentrations were generally higher in adults, the gradient from least to most contaminated sites was greater in chicks for PCBs and DDE. From the least to most contaminated herring gull chick livers, there was a 42.1× increase in PCBs, a 26.4× increase in HG-TEQs, and an 80.3× increase in DDE. In adults, there was a 13.2× increase in PCBs, a 50.4× increase in HG-TEQs, and a 12.5× increase in DDE. PCB, HG-TEQ, and DDE concentrations in livers of chicks from Upper Green Bay were much higher than those from Saginaw Bay, which was the next most contaminated site. Livers from adults from Middle Island in Lake Erie had an extremely high value for calculated HG-TEQs. This value was driven by a high concentration of PCB congener #126.

EROD activity was higher in adults than in chicks (1.8× for minimum values, 1.5× for maximum values). Maximum induction for both chicks and adults occurred at Hamilton Harbour in Lake Ontario. From the lowest to highest values, chicks showed an increase of 11.6× and adults showed an increase of 9.5×. Over the entire exposure range,

mean EROD activity was not correlated with PCBs (r_{11}=-0.12, P=0.72 for chicks, r_{13}=-0.06, P=0.84). However, EROD activity was positively correlated with PCB concentration at lower exposures. Mean EROD activity increased with PCBs up to 1.0 µg/g in chicks (r_7=0.92, P=0.004) and up to 10 µg/g in adults(r_6=0.90, P=0.015). Above these thresholds, mean EROD decreased in chicks and was variable in adults.

Lymphoid Organs

Of the primary lymphoid organs in herring gull chicks, only the thymus showed the predicted inverse relationship with pollution. Although thymus mass did not show any significant relationships to liver concentrations of individual organochlorine contaminants, there was strong evidence that thymus mass decreased as liver EROD increased (P=0.013; Table 3.1; Fig. 3.1). There was one chick from the upper Green Bay colony that had a bursa of Fabricius that was at least three times larger than any other chick. Histological analysis of this bursa revealed a dilated lumen filled with eosinophilic material and bacterial colonies. There was only a thin rim of bursal tissue, and the bursal follicles exhibited fibrosis. This outlier chick was excluded from further statistical analyses. The mass of the bursa of Fabricius did not show the predicted inverse relationships to liver organochlorine contamination or EROD activity (Table 3.1). There was moderate evidence that bursal mass differed among sites ($F_{10,89}$=2.27, P=0.020). An *a posteriori* Jonckheere test gave marginal evidence for a positive association be PCB contamination and bursal mass (J=627, P=0.064 for a two-way test). Hamilton Harbour and Upper Green Bay fell off the trend for the rest of the data (Fig. 3.2). Most chicks from all sites except the Kent Island colony on the Atlantic coast showed evidence of bursitis caused by a parasitic fluke

(*Cotylurus communis*; Ludwig 1982). Only one of ten chicks from Kent Island was infected with a bursal fluke, and the low grade infestation in this chick was not associated with bursitis. Given the rough qualitative scale used to evaluate the bursitis, preliminary analysis revealed no evidence for a correlation between the incidence of bursitis and bursal mass.

There was no evidence that spleen mass was associated with contaminants or EROD in chicks or adults (Tables 3.1 and 3.2). In chicks, there was very strong evidence that spleen mass differed among sites ($F_{10,87}$=5.52, P<0.0001; Fig. 3.3). There was more than a three-fold difference between the highest and lowest mean spleen masses, but these maximum and minimum means occurred at the two reference sites from outside the Great Lakes. Preliminary analysis of histological reports did not show any clear microscopic differences among spleens from different sites that could explain this variation in spleen mass. In adults, there was little or no evidence that spleen mass was influenced by site ($F_{12,129}$=1.41, P=0.17), sex ($F_{1,129}$=0.14, P=0.71), or a site × sex interaction ($F_{12,129}$=1.30, P=0.23; Fig. 3.4). The mean spleen mass for adult herring gulls at all sites was 0.49±0.02 g or 0.047±0.002% of body mass. Preliminary analysis of histological reports revealed that 64% of the spleens contained no abnormalities. Thirty-two percent of the spleens had amyloidosis, the deposition of amorphous, hyaline, eosinophilic protein in or around the blood vessels or basement membranes. This amyloidosis is often associated with antigenic stimulation during immune responses. Preliminary analysis revealed no association between amyloidosis and organochlorine contamination.

Endocrine Organs

There was little or no evidence that adrenal mass was associated with environmental contaminants or liver EROD in either chicks or adults (Tables 3.1 and 3.2). Adrenal mass differed significantly among sites in chicks ($F_{10,87}$=3.07, P=0.0022; Fig. 3.5) and in adults ($F_{12,126}$=3.59, P=0.00013; Fig. 3.6). In chicks, the mean adrenal masses from the two reference sites outside the Great Lakes fell at the high and low ends of the range of means for all sites. In adults, there was little or no evidence that sex ($F_{1,26}$=0.46, P=0.50) or a site × sex interaction ($F_{12,126}$=1.51, P=0.13) influenced adrenal mass.

Thyroid mass clearly increased with contamination in herring gull adults but less clearly in chicks. In adults, there was very strong evidence that thyroid mass increased with liver PCBs (P=0.0006), HG-TEQs (P=0.0002), and EROD activity (P=0.0042; Table 3.2; Fig. 3.6). These positive associations were greatly influenced by the low mean thyroid mass at the Kent Island reference site, which was lower than all of the Great Lakes sites. Jackfish Bay had the highest mean thyroid mass of all the sites at which adult gulls were sampled. There was moderate evidence that a site × sex interaction influenced thyroid mass ($F_{12,133}$=2.10, P=0.033). At all sites other than Jackfish Bay, males tended to have slightly larger thyroids than females, or there was little difference among sexes. However, at Jackfish Bay, females had much larger thyroids than males (0.31% of body mass as compared to 0.19%). The high mass for the five females from Jackfish Bay, which represented half of the sample, greatly increased the mean mass for the site. In herring gull chicks, there was strong evidence that increasing thyroid mass was associated with increasing liver EROD activity (P=0.008; Table 3.2), but thyroid mass did not increase with the concentration of any individual organochlorine in the liver. An *a posteriori* test

indicated that thyroid mass decreased with HG-TEQs (J=835, P=0.0012 for a two-way test).

Relationships among Biomarkers

Pearson's correlation analysis was used to explore relationships among biomarkers (Table 3.3). The objective was to determine whether there were any associations between different immunological and hematological parameters and whether any biochemical biomarkers could serve as surrogates for immunological biomarkers.

There were few associations between organ masses and biochemical biomarkers such as thyroxine, vitamin A, and highly carboxylated porphyrins. In herring gull adults, there was an inverse relationship between thyroid mass and liver retinyl palmitate, the stored form of vitamin A ($P<0.001$). In adults, thyroid mass was not correlated with total plasma thyroxine (P=0.51). Thyroxine was not measured in chicks.

The mass of primary lymphoid organs showed some relationships with white blood cell counts in herring gull chicks. There was no evidence for any correlations between bursal mass and total white blood cells, total heterophils, or total lymphocytes. Total white blood cell counts, total heterophils, or total lymphocytes were not correlated with thymus mass. The heterophil to lymphocyte ratio was negatively correlated with thyroid mass in herring gull chicks (P=0.002) but not in adults (P=0.80).

Discussion

In laboratory rodents and birds, one of the most consistent and sensitive effects of exposure to HAHs is thymic atrophy (Vos and Van Driel-Grootenhuis 1972, Silkworth and

Grabstein 1982, Vos and Luster 1989). This atrophy is characterized by a reduction in the number of lymphocytes in the thymus, primarily in the thymic cortex(Nikolaidis *et al.* 1988a, Andersson *et al.* 1991). Several studies have suggested that TCDD affects the maturation and selection of T lymphocytes in the developing thymus (Greenlee *et al.* 1985, Lundberg *et al.* 1990, Holladay *et al.* 1991), perhaps inhibiting the transition of $CD4^-CD8^+$ cells to $CD4^+CD8^+$ thymocytes (Blaylock *et al.* 1992). The net result, when combined with effects at earlier and later stages in T lymphocyte development (Fine *et al.* 1990, Neubert *et al.* 1991), is suppression of a variety of T cell functions (Vos and Van Driel-Grootenhuis 1972; Sharma *et al.* 1978; Hinsdill *et al.* 1980; Thomas and Hinsdill 1980; Clark *et al.* 1981, 1983; Takagi *et al.* 1987; Neubert *et al.* 1991; Tomar *et al.* 1991).

In the present study, increasing liver EROD activity was associated with thymic atrophy, which is consistent with an Ah-receptor-mediated mechanism. However, there was no association between thymus mass and liver concentrations of PCBs, HG-TEQs, or DDE (Table 3.1; Fig. 3.1). EROD activity is an integrated measure of exposure to a number of contaminants that act via the Ah-receptor. EROD and aryl hydrocarbon hydroxylase (AHH), a related enzyme, have been used as biomarkers for exposure to coplanar HAHs in colonial fish-eating waterbirds from the Great Lakes (Boersma *et al.* 1986, Hoffman *et al.* 1987; Fox 1993), British Columbia (Bellward *et al.* 1990, Sanderson *et al.* 1994), and the Netherlands and Belgium (Murk *et al.* 1992, Van den Berg *et al.* 1994). Contaminants other than HAHs, including polycyclic aromatic hydrocarbons, can induce EROD. In the present study, a positive relationship between PCB contamination and EROD activity held only for PCB exposures up to 1 μg/g in livers of chicks (wet weight) and 10 μg/g in livers of adults. Histological examination of liver tissues in adults revealed no lesions suggesting hepatotoxicity at higher exposures. Analyses of a subset of

the liver tissues from adult gulls in this study showed that low EROD activity was the result of low amounts of enzymatic protein and not inhibition of enzyme activity by alternate substrates such as organochlorines that have accumulated in the liver (S. Kennedy, pers. commun.).

Polymorphism at the *Ah* gene locus makes some strains of laboratory animals more susceptible than others to Ah-receptor-active chemicals, including immunotoxic effects (Silkworth and Grabstein 1982, Silkworth *et al.* 1984, Nagarkatti *et al.* 1984, Kerkvliet *et al.* 1990a,b). It is possible that at highly contaminated Great Lakes sites, Ah-receptor-mediated toxicity has selected for herring gulls that have low Ah receptor activity, leading to lower EROD activity than expected based solely on contamination. Ah receptor activity and exposure to HAHs *both* are important for determining Ah-receptor-mediated effects, including immunotoxicity and EROD activity.

Although thymic mass was not associated with any single organochlorine contaminant in this experiment, the thymic atrophy associated with high EROD activity strongly suggests that the complex mixtures of contaminants in the Great Lakes exert toxic effects on the immune systems of young herring gulls. In chicken embryos, PCB congener #126 induces the activity of EROD in thymic tissue, demonstrating that the thymus is a target organ for Ah-receptors-mediated toxicity (Lorr *et al.* 1992). T lymphocytes perform many key roles in immunoregulation and cytotoxicity, so changes in thymus size and structure could potentially have important impacts on defenses against diseases. Tests of immune function in herring gull and Caspian tern chicks from the Great Lakes have shown strong associations between organochlorine contaminants and suppression of T-cell-mediated immunity (Chapter 4).

The bursa of Fabricius, the primary lymphoid organ for B lymphocyte development in birds, also is sensitive to HAHs during development and growth. The offspring of chicken hens fed diets containing 10 μg/g of various Arochlor (PCB) mixtures had reduced bursal masses even after the hens had been switched to control diets for eight weeks (Harris *et al.* 1976). Although bursal mass was decreased, PCBs did not suppress the antibody response to *Brucella abortus*. Coplanar PCBs and TCDD have produced dose-dependent decreases in the number of viable lymphocytes in the bursae of chicken embryos that developed in eggs injected with HAHs (Nikolaidis *et al.* 1988b, Andersson *et al.* 1991). The activity of the enzyme aryl hydrocarbon hydroxylase (AHH) in the bursa increased in a dose-dependent manner. This enzyme is induced when HAHs bind to the Ah-receptor and initiate transcription of certain genes. AHH activity normally is measured in liver tissue, but its induction in the bursa shows that this organ also is a target organ for this mechanism of toxicity. However, at the same dose of PCB congener #126 in chicken embryos, EROD was approximately four times higher in the thymus than the bursa, suggesting that T-cell-mediated immunity is more sensitive than antibody-mediated immunity to toxic effects mediated by the Ah receptor (Lorr *et al.* 1992). TCDD and 3,3′,4,4′-tetrachloroazoxybenzene decreased the number of lymphocytes in bursae that had been removed from chicken embryos, exposed to either HAH, and implanted into the chorioallantoic membrane of other embryos (Nikolaidis *et al.* 1990). Although the number of lymphocytes in the bursa were decreased, the bursal epithelium showed essentially normal development when examined microscopically.

In the present study, bursal mass did not decrease as liver organochlorine concentrations or liver EROD activity increased. However, the assessment of bursal mass was complicated by the high incidence of fluke infestation (*Cotylurus communis*) at all sites

except Kent Island on the Atlantic coast. Only one of ten chicks from Kent Island was infected with a bursal fluke, and the low grade infestation in this chick was not associated with bursitis. It is possible that the fluke at Kent Island a different species than *C. communis*. While birds are the definitive hosts for some trematodes, the life cycle of these flukes requires snails and sometimes fish as a intermediate hosts. It is probable that the appropriate intermediate host for *C.. communis* was absent or rare in the marine ecosystem at Kent Island, hence the lack of bursitis at this site. At the other sites, alteration of bursal tissues by the presence of the flukes imposed an additional factor besides contaminants to influence bursal growth and development. This fluke has been implicated in the death of herring gull chicks in the Great Lakes (Ludwig 1982).

In the absence of infectious organisms, immunosuppression may cause a decrease in the mass of the spleen as there are fewer functional lymphocytes inside this organ. Indeed, Harris *et al.* (1976) observed decreased spleen masses in the offspring of chicken hens fed PCBs. However, immunosuppression that leads to recurrent infections may result in splenomegaly. Hence, the effect of immunosuppression on spleen mass depends on the presence of infectious organisms, which may vary from site to site in a field study. In this study, there was no evidence for any association between spleen mass and organochlorine contaminants or EROD activity (Table 3.1).

Because of the close ties between the immune and endocrine systems, effects of contaminants on the endocrine system could influence the structure and function of the immune system. Adrenal glucocorticoids such as corticosterone and cortisol have immunosuppressive and anti-inflammatory effects (Schrank *et al.* 1990, Grasman and Scanlon 1995). Environmental contaminants, including HAHs, polycyclic aromatic hydrocarbons, and heavy metals, have been associated with altered adrenal function in

animals (Rattner *et al.* 1984, Hontela *et al.* 1992, 1995). This study revealed no associations between adrenal mass and organochlorine contaminants in herring gull adults or chicks. There were no correlations between immunological or hematological parameters and adrenal mass.

Thyroid mass, histology, and hormones have proven to be responsive biomarkers to contaminants, in particular HAHs. Effects on these parameters have been demonstrated in fish-eating birds from the Great Lakes (Moccia *et al.* 1986, Fox 1993) and the Netherlands (Van den Berg *et al.* 1994), fish from the St. Lawrence River (Hontela *et al.* 1995), and marine mammals from Europe (Brouwer *et al.* 1989). Organochlorine-associated goiter was demonstrated in adult herring gulls from the Great Lakes during the early 1980's (Moccia *et al.* 1986). In the present study, gulls were collected from some of the same sites as this earlier study, and thyroid masses have generally decreased over time (Fox *et al.* in prep.). In the 1990's collections, thyroid mass increased as liver PCBs, HG-TEQs, and EROD activity increased (Table 3.2). The greatest difference in thyroid mass was between Atlantic coast (Kent Island) and inland (Great Lakes and Lake Winnipeg) sites. Within the Great Lakes sites, there was not a clear relationship between thyroid mass and contaminants. The enlarged thyroid glands at Jackfish Bay might be related to nutritional stress related to food shortage (G. Fox, pers. commun.). The effects of contaminants on thyroid mass in chicks were less clear, showing a positive association with liver EROD activity but a negative association with HG-TEQs (Table 3.1).

This study showed that immunological and endocrine organ masses are useful biomarkers for assessing the health of wild birds, although their interpretation is confounded by several factors. Many laboratory studies have documented reduced masses of the thymus and bursa of Fabricius after developmental exposure to HAHs. In these

laboratory studies, contaminant exposure and nutrition were strictly controlled, and the presence of infectious diseases was reduced or eliminated. In wild birds, pre- and post-hatch exposure may differ depending local food supplies. Infectious diseases may increase the mass of immunological organs, especially in immunosuppressed birds. In this experiment, the presence of flukes in the bursae of most birds obscured the potential effects of contaminants. Examination of immune organ masses in developing embryos would eliminate the problems of changing exposure and nutrition and the presence of many infectious diseases. Despite these confounding factors in the present study, an association between thymic atrophy and increasing liver EROD activity suggested that such contamination may in fact alter immunological defenses against infectious agents. As in the National Toxicology Program's procedures for screening immunotoxic chemicals in laboratory animals (Luster *et al.* 1988b, 1994), immune organ masses are important variables that should be measured along with more sophisticated tests of immune function to assess the immunotoxic risk presented by pollutants in wild animals.

Table 3.1. Effects of contaminants on masses of immunological and endocrine organs in herring gull chicks from the Great Lakes, Lake Winnipeg, and the Atlantic coast during 1991 and 1992. Organochlorine contaminants were measured in pooled liver samples.

Dependent Variable (Organ Mass Expressed as % of Body Mass)	Independent Variable	Predicted Trend[1]	Actual Trend	Test for Ordered Alternatives	
				Jonckheere Statistic	P-Value
Thymus	PCBs	-	+	-164	0.70
	HG-TEQs	-	+	-466	0.95
	DDE	-	+	-196	0.74
	EROD	-	-	**610**	**0.013**
Bursa of Fabricius	PCBs	-	+	-655.0	0.97
	HG-TEQs	-	+	-279	0.78
	DDE	-	+	-387	0.87
	EROD	-	+	-105	0.62
Spleen	PCBs	+/-	+	433	0.084
	HG-TEQs	+/-	-	415	0.18
	DDE	+/-	+	573	0.075
	EROD	+/-	-	577	0.061
Adrenal Gland	PCBs	+/-	+	169	0.098
	HG-TEQs	+/-	+	-199	0.60
	DDE	+/-	+	81	0.16
	EROD	+/-	-	-3	0.24
Thyroid Gland	PCBs	+	+	77	0.13
	HG-TEQs	+	-	-1405	1.0
	DDE	+	+	-169	0.35
	EROD	+	+	**591**	**0.008**

[1] "+/-" means that the direction of the trend could not be predicted *a priori*.

Table 3.2. Effects of contaminants on immune and endocrine organ masses in herring gull adults from the Great Lakes, Lake Winnipeg, and the Atlantic coast during 1991 and 1993. Organochlorine contaminants were measured in pooled liver samples.

Dependent Variable (Organ Mass Expressed as % of Body Mass)	Independent Variable	Predicted Trend[1]	Actual Trend	Test for Ordered Alternatives	
				Jonckheere Statistic	P-Value
Spleen	PCBs	+/-	-	785	0.15
	HG-TEQs	+/-	-	289	0.47
	DDE	+/-	+	221	0.55
	EROD	+/-	+	381	0.41
Adrenal Gland	PCBs	+/-	-	-215	0.43
	HG-TEQs	+/-	-	-361	0.58
	DDE	+/-	+	-713	0.99
	EROD	+/-	-	-403	0.61
Thyroid Gland	PCBs	+	+	**1449**	**0.0006**
	HG-TEQs	+	+	**2565**	**0.0002**
	DDE	+	+	71	0.16
	EROD	+	+	**1269**	**0.0042**

[1] "+/-" means that the direction of the trend could not be predicted *a priori*.

Table 3.3. Pearson's correlation analysis exploring relationships among immune and endocrine organ sizes and other biomarkers in herring gull chicks and adults from the Great Lakes, Lake Winnipeg, and the Atlantic coast during 1991-93.

Relationship	Age	Variables		r	N	P
Organ Masses and Biochemical Biomarkers	Adult	Thyroid Mass	Retinyl Palmitate	-0.37	160	<0.001
	Chick	Thyroid Mass	Retinyl Palmitate	0.18	89	0.10
	Adult	Thyroid Mass	Thyroxine	0.060	120	0.51
Hematological Variables and Organ Masses	Chick	H/L Ratio[1]	Thyroid Mass	-0.32	91	0.0002
	Adult	H/L Ratio	Thyroid Mass	0.021	151	0.80

[1] Heterophil/Lymphocyte Ratio

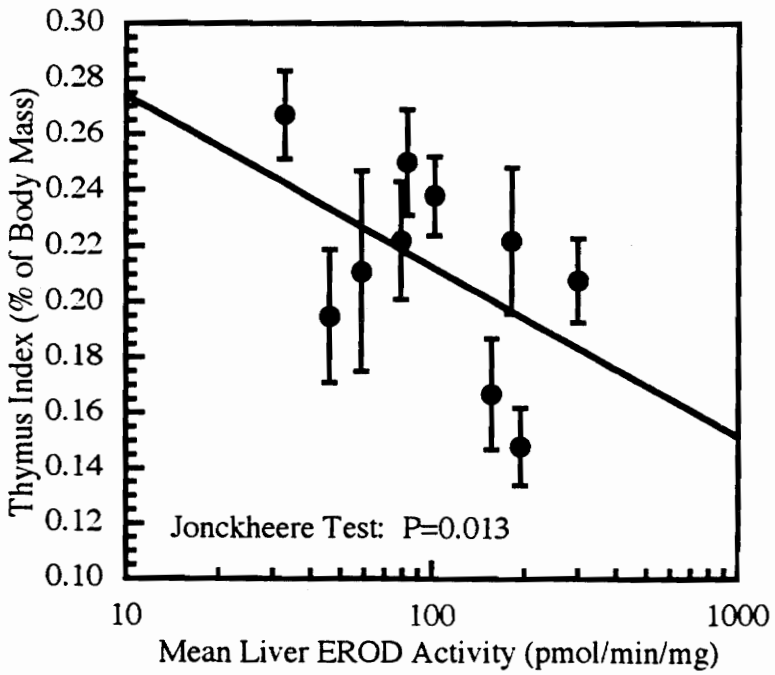

Fig. 3.1. Relationship between liver EROD activity and thymus mass in herring gull chicks from the Great Lakes and the Atlantic coast during 1991 and 1992. Error bars indicate one standard error of the mean.

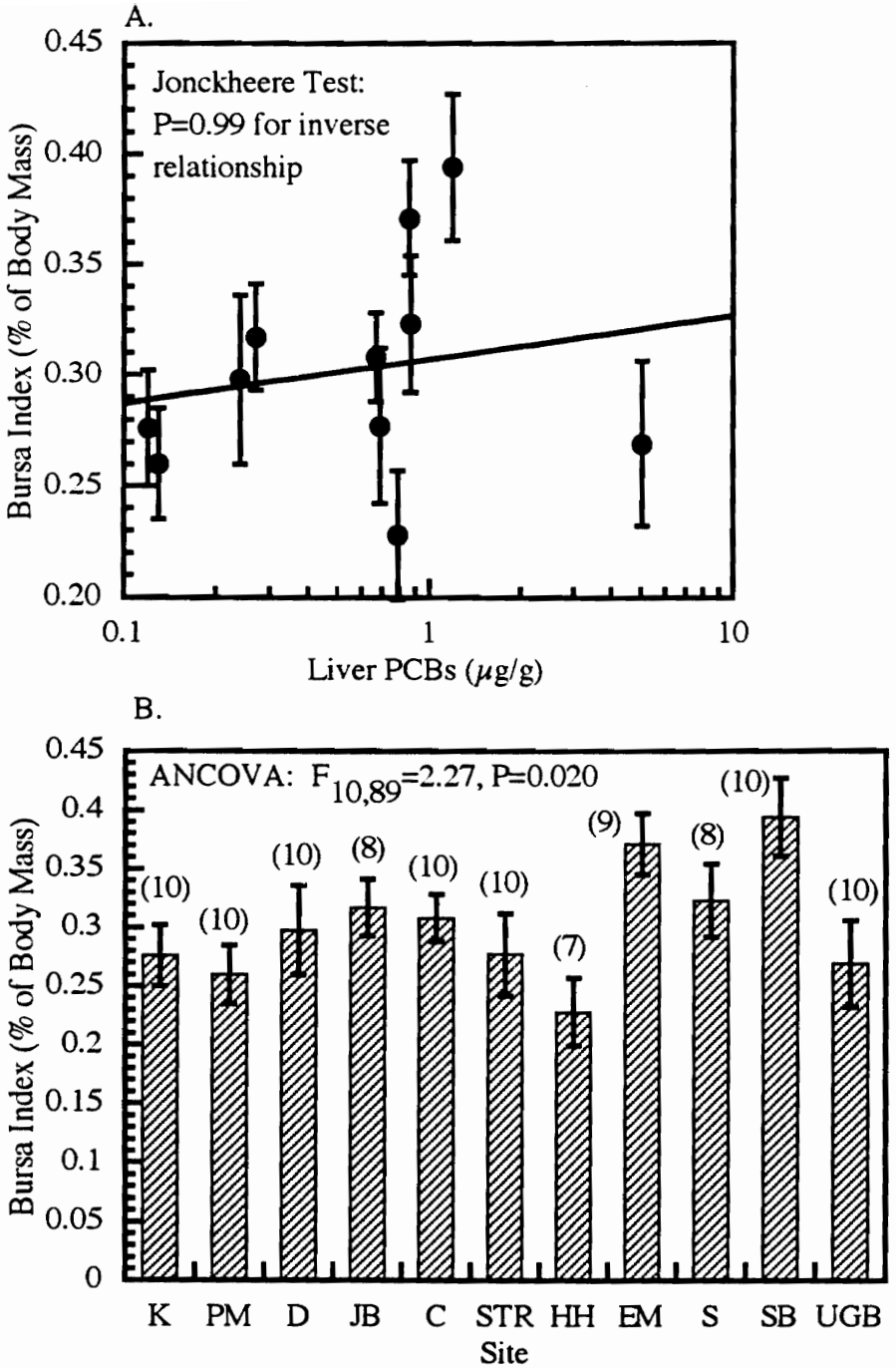

Fig. 3.2. Spatial differences in mean bursal mass and the relationship to liver PCB concentrations in herring gull chicks from the Great Lakes, Lake Winnipeg, and the Atlantic coast during 1991 and 1992. Error bars indicate one standard error of the mean. Numbers in parentheses indicate sample sizes. For ANCOVA, body mass was used as the covariate.

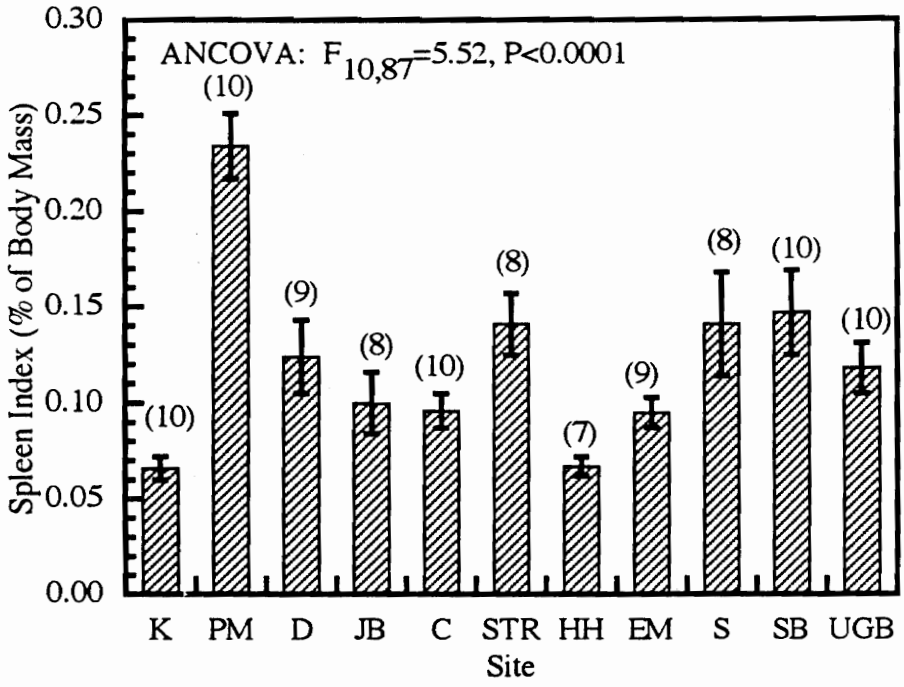

Fig. 3.3. Spatial differences in mean spleen mass in herring gull chicks from the Great Lakes, Lake Winnipeg, and the Atlantic coast during 1991 and 1992. Error bars indicate one standard error of the mean. Numbers in parentheses indicate sample sizes. For ANCOVA, body mass was used as the covariate.

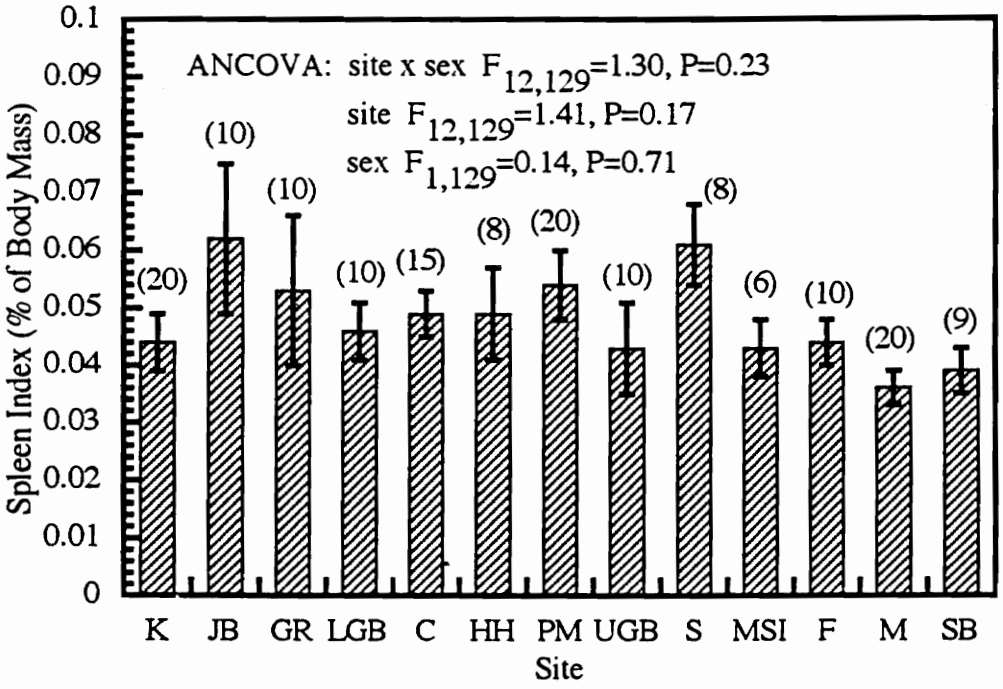

Fig. 3.4. Spatial differences in mean spleen mass in herring gull adults from the Great Lakes, Lake Winnipeg, and the Atlantic coast during 1991 and 1993. Error bars indicate one standard error of the mean. Numbers in parentheses indicate sample sizes. For ANCOVA, body mass was used as the covariate.

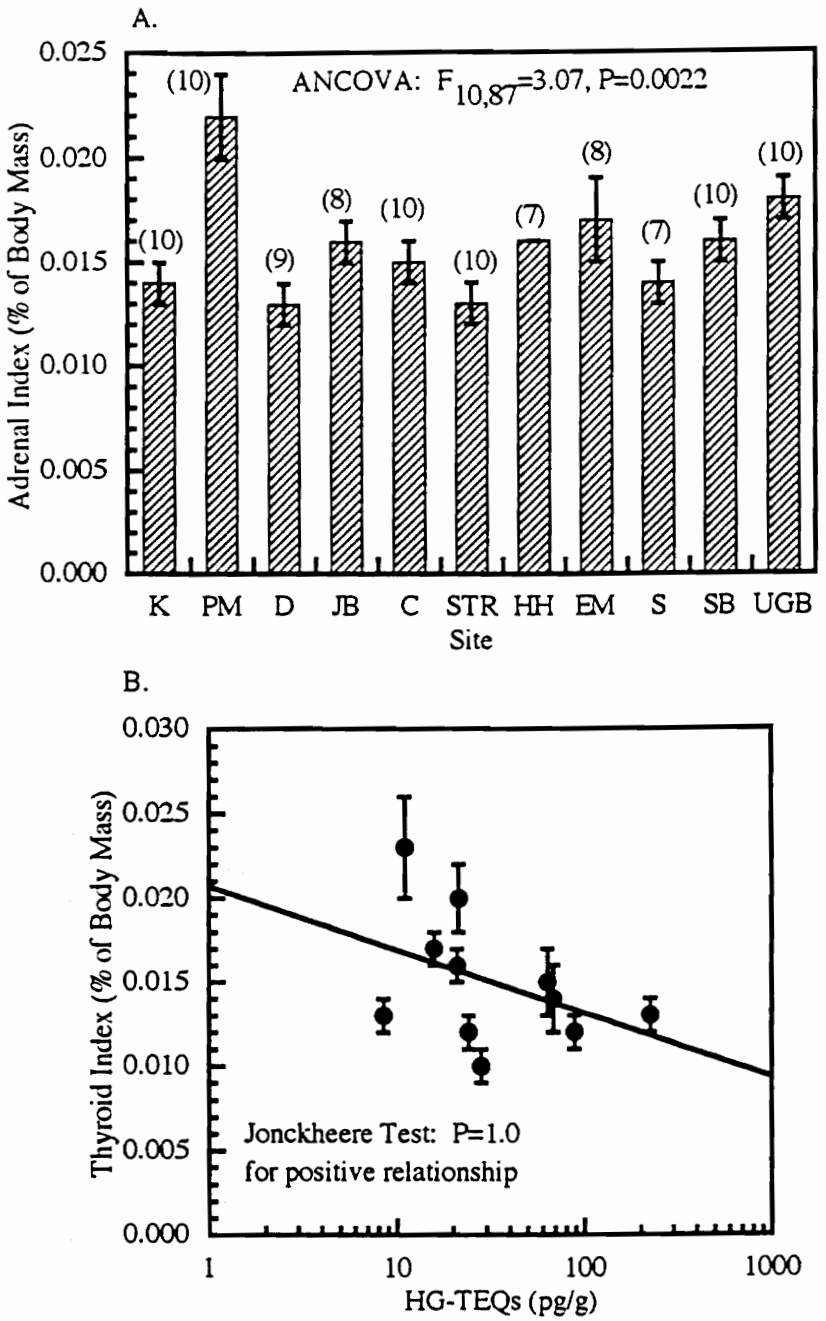

Fig. 3.5. Spatial differences in mean adrenal mass and the relationship between mean thyroid mass and liver HG-TEQ concentrations in herring gull chicks from the Great Lakes, Lake Winnipeg, and the Atlantic coast during 1991 and 1992. Error bars indicate one standard error of the mean. Numbers in parentheses indicate sample sizes. For ANCOVA, body mass was used as the covariate.

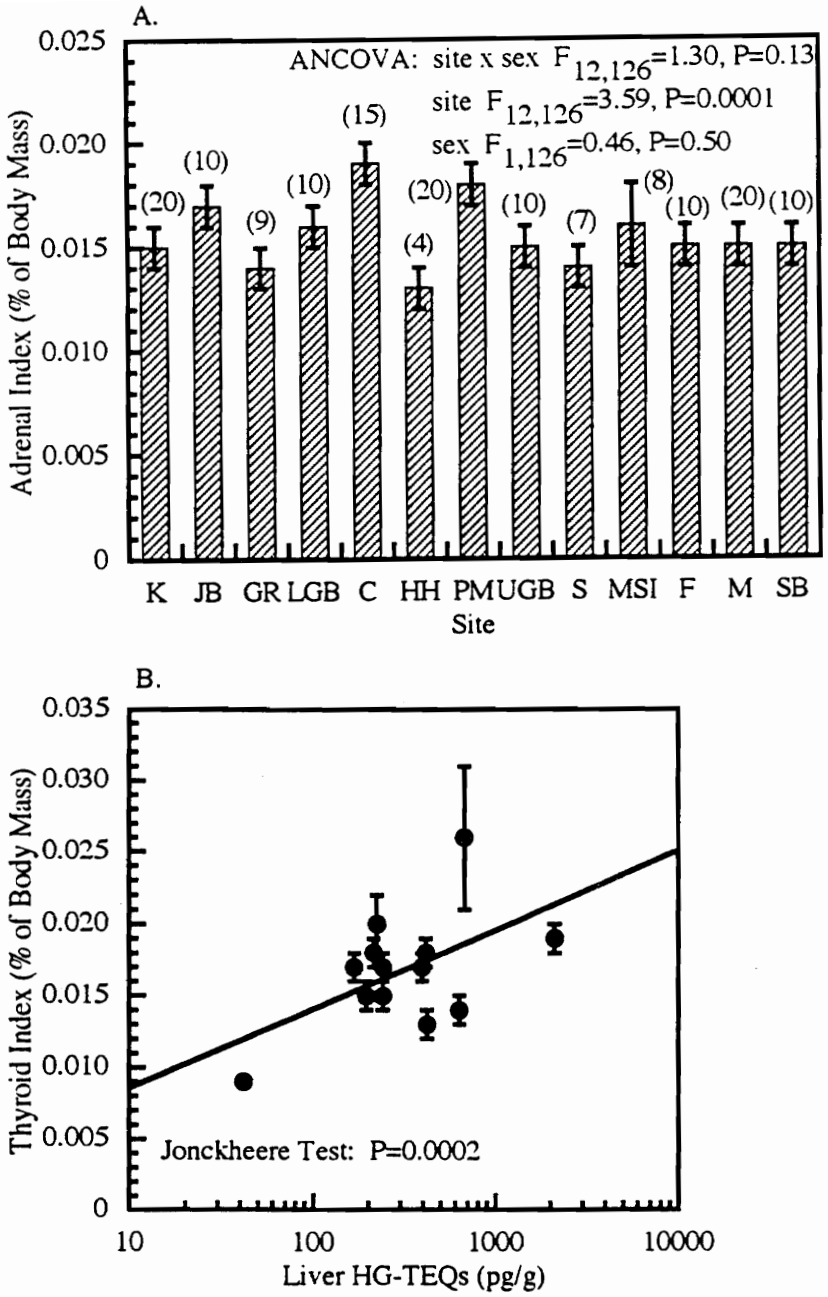

Fig. 3.6. Spatial differences in mean adrenal mass and the relationship between mean thyroid mass and liver PCB concentrations in herring gull adults from the Great Lakes, Lake Winnipeg, and the Atlantic coast during 1991 and 1993. Error bars indicate one standard error of the mean. Numbers in parentheses indicate sample sizes. For ANCOVA, body mass was used as the covariate.

Chapter 4. Immunological Biomarkers and Immunosuppression by Organochlorine Contaminants in Fish-Eating Birds of the Great Lakes

Summary

This study investigated the association between environmental contaminants and immune function in fish-eating birds of the Great Lakes and evaluated the use of immunological, hematological, and biochemical measures as biomarkers of contaminant-associated health effects in wild birds. A primary focus was to study immunotoxic effects in young birds since the developing immune system is particularly sensitive to environmental contaminants. During 1991-1994, specific immune functions and general immunological, hematological, and biochemical parameters were measured in herring gull (*Larus argentatus*) and Caspian tern (*Sterna caspia*) chicks. Study sites were chosen to provide a wide range of organochlorine contamination caused primarily by PCBs. In gull and tern chicks, T cell function was assessed using the phytohemagglutinin skin test. In both species, T-cell-mediated immunity showed a strong decrease as organochlorine concentrations in eggs increased. The suppression of T-cell-mediated immunity observed in this field study was consistent with the documented effects of developmental exposure to PCBs and TCDD in laboratory animals. It also was consistent with thymic atrophy observed in herring gull chicks at sites with higher mean liver EROD activity, an integrated

measure of exposure to contaminants that act via the Ah receptor (Chapter 3). In both species, chicks exhibited biologically significant differences in antibody production among sites, but relationships to contaminants or other factors were unclear. In published studies with laboratory rodents, PCBs most consistently suppress antibody-mediated immunity at high acute doses rather than at the chronic exposures during development that were observed in this field study. In gull and tern chicks, plasma retinol (vitamin A) concentrations decreased as egg organochlorine concentrations increased. Organochlorine contamination in the herring gulls and Caspian terns of the Great Lakes results in decreased T-cell-mediated immune function, and immunological and hematological variables are useful biomarkers for assessing the effects of contaminants on wild birds.

Introduction

During recent years, numerous studies have demonstrated that environmental contaminants can suppress immunological function and increase susceptibility to infectious diseases in laboratory animals (Koller 1979, Caren 1981, Luster *et al.* 1987, Vos *et al.* 1989, Wong *et al.* 1992, Holladay and Luster 1994, Pruett 1994). Often these chemicals act at low concentrations, especially in developing or growing animals (Takagi *et al.* 1987, Holladay and Luster 1994). Such perinatal effects often are more persistent than acute effects in adults. These laboratory effects have raised concerns about potential impacts on wildlife and humans. However, few immunotoxicological investigations have studied free-living wildlife. This study investigated the association between environmental contaminant exposure and immune function in fish-eating birds of the Great Lakes.

Environmental contaminants, especially organochlorines such as polychlorinated biphenyls (PCBs) and 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-ethane (DDT), have caused

numerous physiological, reproductive, and behavioral problems in Great Lakes colonial waterbirds during the last 30 years (Peakall *et al.* 1980; Mineau *et al.* 1984; Fox and Weseloh 1987; Peakall and Fox 1987; Gilbertson 1988, 1989; Peakall 1988; Kubiak *et al.* 1989; Colborn *et al.* 1990; Fox *et al.* 1991; Gilbertson *et al.* 1991; Government of Canada 1991; Fox 1993; Mora *et al.* 1993). The high position of fish-eating birds in the food web exposes them to elevated levels of contaminants that biomagnify. Although reproductive success has recovered in many areas following restriction of many chemicals, significant impacts still occur at highly contaminated sites. The important symptoms include high embryonic mortality, subcutaneous, pericardial, and peritoneal edema, congenital deformities, growth retardation, hepatomegaly, liver necrosis, and liver porphyria (Gilbertson 1989). Gilbertson *et al.* (1991) named this set of symptoms Great Lakes embryo mortality, edema, and deformities syndrome (GLEMEDS). GLEMEDS is similar to chick edema disease, a syndrome found in chickens exposed to PCBs. In wild birds, GLEMEDS has been linked to halogenated aromatic hydrocarbons (HAHs), which include PCBs, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and 2,3,7,8-tetrachlorodibenzo-*p*-furan (TCDF). HAHs exert many of these toxic effects by binding to an intracellular protein called the *Ah*-receptor. The HAH-*Ah*-receptor complex then influences the expression of many genes, including those for the detoxification enzymes in the cytochrome P450 1A1 (CYP 1A1) family (Safe 1990).

In laboratory animals, including chickens with chick edema disease, HAHs suppress several types of immune responses. T lymphocytes develop in the thymus gland. They are responsible for regulating many immune responses and for attacking virus-infected and malignant cells. In developing animals, low levels of HAHs cause thymic atrophy (Vos and Van Driel-Grootenhuis 1972, Silkworth and Grabstein 1982, Nikolaidis

et al. 1988a,b, Vos and Luster 1989, Andersson *et al.* 1991). Toxic effects have been observed at many stages of T lymphocyte development, including the prothymocyte stage in the bone marrow (Fine *et al.* 1990), thymocyte selection in the thymus (Greenlee *et al.* 1985, Lundberg *et al.* 1990, Holladay *et al.* 1991, Blaylock *et al.* 1992), and the mature T lymphocyte stage in the blood (Neubert *et al.* 1991). As a result, a number of T cell functions are suppressed (Vos and Van Driel-Grootenhuis 1972; Sharma *et al.* 1978; Hinsdill *et al.* 1980; Thomas and Hinsdill 1980; Clark *et al.* 1981, 1983; Takagi *et al.* 1987; Tomar *et al.* 1991). B lymphocytes develop in the bursa of Fabricius in birds and the bone marrow in most mammals. B lymphocytes respond to foreign antigens by producing specific antibodies, which help identify and destroy invading microorganisms. High doses of HAHs suppress antibody production in laboratory animals (Sharma *et al.* 1978; Thomas and Hinsdill 1978; Hinsdill *et al.* 1980; Clark *et al.* 1981, 1983; Silkworth and Grabstein 1982; Silkworth *et al.* 1984; Luster *et al.* 1988a; House *et al.* 1990; Kerkvliet *et al.* 1990b). HAHs appear to exert their toxic effects on the immune system primarily through *Ah*-receptor-dependent mechanisms (Silkworth and Grabstein 1982, Silkworth *et al.* 1984, Nagarkatti *et al.* 1984, Safe 1984, Luster *et al.* 1987, Vos and Luster 1989, Kerkvliet *et al.* 1990a,b), although *Ah*-receptor-independent mechanisms also are involved (Kerkvliet *et al.* 1990a,b, Davis and Safe 1991, Morris *et al.* 1992).

In addition to these direct effects on the immune system, HAHs also significantly reduce circulating levels of vitamin A (retinol; Zile 1992) and thyroxine. Both of these substances are important for proper immune function (Friedman and Sklan 1989, Bruns and Webb 1990, Haddad and Mashaly 1991). Various forms of vitamin A occur at low concentrations in herring gulls from some highly contaminated Great Lakes colonies (Government of Canada 1991). Brouwer *et al.* (1989) fed PCB-contaminated fish from the

Wadden Sea to common seals and found that these seals had lower plasma retinol, triiodothyronine, and total and free thyroxine. They concluded that PCBs might cause immunosuppression in wild seals through their effects on retinol homeostasis.

HAHs may have contributed to epizootics in marine mammals exposed to high concentrations of organochlorine pollutants. The disease outbreaks have occurred in beluga whales (*Delphinapterus leucas*) in the St. Lawrence Estuary (Martineau *et al.* 1988), California sea lions (*Zalophus californianus*) on San Miguel Island (Gilmartin *et al.* 1976, Barinaga 1988), common seals (*Phoca vitulina*) in Europe (McGourty 1988), and bottlenose dolphins (*Tursiops truncatus*) in the Atlantic Ocean (Lahvis *et al.* 1995). Several recent studies have lent support to this hypothesis. Harbor seals fed organochlorine-contaminated herring from the Baltic Sea had altered white blood cell counts and immune function (DeSwaart *et al.* 1994, Ross *et al.* 1995). Lahvis *et al.* (1995) found that T-cell immunity was inversely related to PCB and DDE contamination in male bottlenose dolphins from the Atlantic Ocean. At highly contaminated sites in the Great Lakes, double-crested cormorants (*Phalacrocorax auritus*) have an abnormally high incidence of eye infections associated with *Pasteurella multocida* (Ecological Research Services 1991). In laboratory animals, HAHs can suppress immune function by a number of mechanisms and increase susceptibility to bacteria (Thomas and Hinsdill 1978, Vos *et al.* 1978, Hinsdill *et al.* 1980), viruses (Friend and Trainer 1970, Imanishi *et al.* 1980, Vos and Luster 1989, House *et al.* 1990), and protozoan parasites (Vos and Luster 1989). Children who have been exposed perinatally to PCBs, PCDDs, and PCDFs in arctic Quebec experienced an increased incidence of middle ear infections during the first year of life (Dewailly *et al.* 1993).

The application of immunological tests to wild species presents many logistical difficulties. Many sophisticated tests of immune function that involve cell culture or flow

cytometry often are not practical for widespread field studies because it is difficult to transport fresh blood samples to a distant laboratory or sophisticated equipment into the field. Developing these cell culture techniques for wild species often is time consuming. Wild animals may not be readily available for testing except during brief time periods such as the breeding season. Measurement of immune organ masses, histology, and white blood cell counts can provide important information about immunocompetence and could be included in immunotoxicological assessments. However, it is possible for immune organs to have normal size and for cells to be normal in number but still have abnormal function. Usually, a battery of structural and functional variables should be studied to survey several aspects of immunocompetence. Optimally, immunological tests for field research should be sensitive, reproducible, simple, and relatively inexpensive.

This study examined the association between environmental contaminants and immune function in herring gulls and Caspian terns throughout the Great Lakes region. The herring gull was chosen because it is the most frequently used and best understood vertebrate bioindicator species in the Great Lakes. The Caspian tern was chosen because its high sensitivity and high exposure to contaminants have produced significant reproductive and population-level effects (Ludwig *et al.* 1993, Mora *et al.* 1993). The objectives of the study were to determine whether contaminant-associated immunosuppression occurs in these birds and to evaluate several immunological biomarkers for contaminant effects. This study employed two specific *in vivo* tests of immune function: the phytohemagglutinin (PHA) skin test for T-cell-mediated immunity and the sheep red blood cell (SRBC) hemagglutination test for antibody-mediated immunity. It also measured general biomarkers such as white blood cell counts, plasma retinol (vitamin A), and thyroxine.

Materials and Methods

Sampling Design

During 1992, herring gull chicks were sampled across a gradient of contamination at the following four sites within the Great Lakes and one site outside the Great Lakes (Tables 4.1 and 4.2; Fig. 4.1): 1) Little Charity Island in Saginaw Bay, Lake Huron; 2) Bird and Anchor Islands in the North Channel, Lake Huron; 3) Monroe, on the western shore of Lake Erie; 4) Hamilton Harbour in eastern Lake Ontario; and 5) Pony Island on the north end of Lake Winnipeg, Manitoba. The North Channel and Lake Winnipeg sites were chosen as reference colonies. During 1993, herring gulls were sampled at one reference site, Bird/Anchor Islands in the North Channel, and at one highly contaminated site, Little Charity Island in Saginaw Bay. During 1994, herring gulls were again sampled at Little Charity Island. During 1992, Caspian tern chicks were sampled across a gradient of contamination at the following five sites within the Great Lakes (Tables 4.1 and 4.2; Fig. 4.1): 1) Gravelly Island in upper Green Bay, Lake Michigan; 2) High Island in northern Lake Michigan; 3) Elm Island in the North Channel, Lake Huron; 4) Confined Disposal Facility in southern Saginaw Bay, Lake Huron; 5) Pigeon Island in eastern Lake Ontario. Elm Island was chosen as a reference colony because of its historically low organochlorine contamination. During 1993 and 1994, Caspian terns were sampled at one reference site, Elm Island in the North Channel, and at one highly contaminated site, the Confined Disposal Facility in Saginaw Bay. Because of logistical difficulties, not all variables were measured at all sites (Table 4.1).

At eight of ten sites, enclosures were erected during mid-incubation. The fences were constructed of plastic mesh (1 × 2 cm) and approximately 1 m high. The fences were placed around groups of 10-20 herring gull nests or 30-40 Caspian tern nests. Usually two or three enclosures were erected at each site. Chicks hatched in the enclosures and were confined until fledging or until the enclosures were removed. Chicks were banded with standard US Fish and Wildlife Service leg bands for individual identification. At two of ten sites, rocky ground prevented the construction of enclosures, so chicks were captured and released in thick vegetation. Immune function tests were initiated on 35-50 chicks at each site. Blood smears, retinol concentrations, and thyroxine concentrations were assessed for 10-20 chicks per site.

Immune function tests were initiated on three week old chicks. Chick age was based on estimated hatch times for the colony and body size measurements for individual chicks. Target body size for herring gull chicks was based on the following criteria a body mass of 400-700 g and a wing chord of 130-200 mm. Criteria for Caspian terns were a body mass of 450-550 g and a wing chord of 130-200 mm. The PHA skin test and SRBC hemagglutination test were initiated on 3 week old chicks (Day 0), and the PHA skin test was completed the following day (Day 1). Also on Day 1, a 4 ml blood sample was drawn for a blood smear and the measurement of retinol and thyroxine. Since antibody titers peak around 6 days after antigen injection in these species, a second 4 ml blood sample was collected on Day 6, or in some cases Day 7 or 8. Plasma was preserved in liquid nitrogen until antibody analysis.

Growth and Reproductive Success

Body size was measured in the same chicks at 3 and 4 weeks of age. Reproductive success was estimated by dividing the number of live chicks (3 or 4 weeks old) in each enclosure by the number of active nests. Most studies assess reproductive success based on 3 week old chicks since it is assumed that most chick mortality occurs before 3 weeks of age in these two species (Gilman *et al.* 1977, Ludwig *et al.* 1993), but the sampling protocol for this study allowed this assumption to be tested by counting 3 and 4 week old chicks.

Functional Tests for Immunocompetence

The PHA skin test for T-cell-mediated immunity was conducted following the procedures of Grasman and Scanlon (1995) using a dose of 0.1 ml of a 1 mg/ml PHA-P (Sigma, St. Louis, MO) dissolved in phosphate buffer saline (PBS). Feathers were plucked from both wing webs. One wing web was injected with PHA while the other received a placebo injection of PBS alone. The thickness of each wing web was measured to the nearest 0.05 mm immediately before and 24±3 hours after the injections using a pressure-sensitive caliper with a low-tension spring that did not crush the skin (Dyer Co., Lancaster, PA). A stimulation index (SI) was calculated as the change in the thickness of the PHA-injected wing web minus the change in thickness of the PBS-injected wing web.

The SRBC hemagglutination test was initiated at the same time as the PHA skin test. Chicks were injected via the wing vein with 0.1 ml of a 1% SRBC suspension in sterile saline. Blood plasma samples were collected from chicks 5-7 days after SRBC injection because antibody titers peak in gulls and terns at approximately 6 days post-

immunization (K. Grasman, unpublished data). Total (IgM + IgG) and 2-mercaptoethanol-resistant (IgG) antibody activities were measured by the microtiter method of Gross and Siegel (1980, 1981) using 0.25% SRBC. SRBC from the same sheep (Colorado Serum Co, Denver, CO) were used for all injections and assays during 1992 and 1993. After this sheep died, SRBC were obtained from a sheep of the same age and flock as the first sheep.

General Immunological and Hematological Biomarkers

On Days 1 and 6, a 4 ml blood sample was drawn from the wing vein using a 22 gauge needle and Vacutainer® tubes containing EDTA (Beckton Dickinson, Rutherford, NJ). Within 5 hours after blood collection, two blood smears were made from the blood collected in EDTA. The blood was centrifuged at 2575×g for 5 minutes, and the plasma was stored on liquid nitrogen. Blood drawn on Day 1 was used for retinol and thyroxine analysis and that drawn on Day 6 was used for antibody analysis. PCV also was measured on Day 1 by centrifuging microhematocrit tubes for 5 minutes at 5125×g for 5 minutes.

Blood smears were fixed with methanol to preserve the cells until staining. Smears were stained with Wright stain (Accustain™, Sigma, St. Louis, MO) using 100% stain for 30 seconds and then diluting with an equal volume of distilled water for 90 seconds. Smears were rinsed with distilled water and allowed to air dry. Two hundred white blood cells were counted and classified using oil immersion microscopy at 1000× magnification.

Plasma retinol was extracted from 100 µl of plasma after the addition of 75 ng of retinyl acetate in 15 µl of 2-propanol as an internal standard. Retinol-protein complexes were dissociated by vigorous shaking after the addition of 200 µl of acetonitrile. The retinol was extracted with successive 4 and 1 ml volumes of hexane. After shaking for 5 minutes, the organic and aqueous phases were separated by centrifugation at 1500 rpm for

5 minutes at 4 °C. The organic phase was dried under nitrogen, reconstituted with 500µl of methanol, and filtered through a 0.2 µm PVDF filter disk. The extract was analyzed by reverse-phase HPLC using an ODS spheri-5 guard column (Brownlee) and a 15 cm long, 5 µm ODS Zorbax (DuPont) analytical column. Methanol (100%) at a flow rate of 1 ml/min was used to separate the retinoids in the plasma extract in less than 6 minutes. Either fluorescence (ex: 336 nm; em: 480 nm) or UV-visible (326 nm) was used to detect the retinoids. The detection limit for retinol in plasma was 5 µg/l.Total plasma thyroxine was measured using a solid phase enzyme assay (veterinary modification of the EZ Bead T4 Test, Immunotech Corp, Boston, MA).

Organochlorine Analysis

The 12 eggs collected from each site were pooled for organochlorine analysis by the analytical services laboratory at the National Wildlife Research Centre of the Canadian Wildlife Service following the methods of Peakall *et al.* (1986). PCB residues are reported as the sum of the following 42 PCB congeners: IUPAC nos. 28, 31, 42, 44, 49, 52, 60, 64, 66, 70, 74, 87, 97, 99, 101, 105, 110, 118, 128, 129, 137, 138, 141, 146, 149, 151, 153, 158, 170, 171, 172, 174, 180, 182, 183, 185, 194, 195, 200, 201, 203, and 206. Nonortho PCB congeners (IUPAC nos. 37,77,126, and 169) and all 2,3,7,8-substituted polychlorinated dibenzo-dioxin (PCDD) and polychlorinated dibenzo-furan (PCDF) congeners also were measured in pooled egg samples by the analytical services laboratory of the National Wildlife Research Centre of the Canadian Wildlife Service. Pooled egg samples were dried with anhydrous sodium sulphate and ground into a fine powder. An open chromatographic column wet-packed with multiple absorbents (anhydrous sodium sulphate, deactivated silica, sulfuric acid on silica, activated silica, and sodium hydroxide

on silica) was used for the initial extraction and cleanup. The sample was spiked with a ^{13}C PCDD mixture (Cambridge Isotope Laboratories) and a ^{13}C PCB 77, 126, and 169 mixture (Wellington Isotope Laboratories) to determine the degree of analyte loss during sample workup. The column was eluted using 1:1 dicloromethane/hexane, and the eluted extract was concentrated by evaporation. A liquid chromatograph (FMS Systems Inc.) with a carbon column was used for further cleanup and trace enrichment. The carbon column was flushed with dichloromethane and backflushed with toluene. The eluent was concentrated by evaporation, and the solvent was changed to hexane. After concentration by evaporation, the extract was cleaned up and separated on a deactivated Florisil column wet packed in hexane. The column was first eluted with 1:20 dichloromethane/hexane for nonortho PCB analysis. The column was then eluted with dichloromethane to produce a second fraction that second fraction was cleaned up on a wet packed, activated basic alumina column (Fisher Scientific, Pittsburgh, PA). The alumina column was eluted with 1:50 dichloromethane:hexane to produce a fraction containing residual PCBs and other organochlorines. The column was then eluted with 1:1 dichloromethane:hexane to produce a fraction for PCDD/PCDF analysis. A Hewlett-Packard 5971A GC/MSD was used to separate and quantify nonortho PCB, PCDD, and PCDF congeners. Detection limits were 75 pg/g for nonortho PCB congeners and approximately 0.3-2.7 pg/g for PCDD and PCDF congeners.

Because different HAH congeners have different relative toxicities, the total biological activity of a complex mixture of congeners cannot be estimated by adding the concentrations of the individual congeners. Furthermore, the relative toxicities of different congeners vary from species to species. Kennedy *et al.* (1994) used *in vitro* induction of EROD activity in primary hepatocyte cultures from 26 day old herring gull embryos to

compare the relative toxicities of TCDD, TCDF, and various PCBs. Based on the EC_{50} for EROD induction, different HAH congeners could be compared to TCDD, the most toxic congener. The following herring gull-specific toxicity equivalency factors (TEFs) were generated: TCDD = 1.0; TCDF = 0.9; PCB congener # 169 = 0.07, PCB congener #126 = 0.06; PCB congeners # 77, #105, #118=0. Multiplying the concentration of each congener by its TEF and then summing the products gave an estimate of the total dioxin-like toxicity of the mixture for herring gulls. This estimate was called HG-TEQs, which stands for herring gull-specific TCDD-equivalents.

Bioassays for TCDD-Equivalents

A chicken embryo hepatocyte bioassay was used to measure the total dioxin-like activity of pooled egg samples. Organochlorines were extracted from pooled egg samples following the procedures of Norstrom *et al.* (1990). The chicken hepatocyte bioassay for EROD induction was carried out in the laboratory of Dr. Sean Kennedy at the National Wildlife Research Centre of the Canadian Wildlife Service following the procedures of Kennedy *et al.* (1993). Based on the *in vitro* induction of ethoxyresorufin-O-deethylase (EROD) in chicken hepatocytes, this bioassay compares the potency of a mixture of HAHs to that of a TCDD standard. The resulting measure of TCDD-like toxicity was designated as chicken bioassay TEQs (C-TEQs), which stands for TCDD toxic equivalents as determined by the chicken bioassay.

Statistical Analyses

The primary goal of this investigation was to determine whether there was an association between the degree of organochlorine contamination and immune function in fish-eating birds of the Great Lakes. The purpose was to determine whether intercolony differences in immunological variables could be explained by exposure-response relationships. Statistical methods were needed to determine the probability that the spatial patterns in response variables were associated with different pollutants as opposed to being caused by random events. The purpose was not to show what percent of variability in immunological responses could be explained statistically by particular chemicals. Because detailed organochlorine analyses are expensive and require large volumes of blood, contaminant concentrations were not measured in individual chicks. However, chemical analysis of pooled egg samples from each site allowed the sites to be ranked on the basis of concentrations of different contaminants.

Statistical methods were chosen to meet this primary goal of associating immune function with pollutants and to work within the constraints of pooled chemical analyses. Regression analysis was not appropriate because of its emphasis on explaining variability using linear models and its need for chemical analyses on individual birds. Although analysis of variance (ANOVA) could detect spatial differences in immune responses among sites, it was not appropriate because it could not be used to look for associations across gradients of contamination.

The Jonckheere test for ordered alternatives was used as the primary statistical method of testing specific hypotheses about contaminant-associated immunosuppression. This test fit the purpose and design of the study as well as the constraints on chemical analysis (Hollander and Wolfe 1973). The null hypothesis for this nonparametric measure

of exposure-response states that there is no difference among the central tendencies from different sites (H_O: $\mu_1 = \mu_2 = \mu_3 = ... = \mu_n$). The alternative hypothesis is that there is a monotonic trend (not necessarily linear) in the data based on *a priori* information (H_A: $\mu_1 \geq \mu_2 \geq \mu_3 ... \mu_n$, where at least one of the inequalities is a strict inequality). In this study, laboratory studies or other field data were used to predict the effects of various contaminants on each response variable. Inverse relationships to contamination were predicted for the PHA skin response, antibody responses, plasma retinol concentrations, and thyroxine concentrations. The direction of the trends for PCV and white blood cell counts could not be predicted from laboratory data, so a two-way test was performed by running the Jonckheere test in both directions and doubling the P-value for the most significant trend. Egg concentrations of total PCBs, DDE, chicken TEQs and, in herring gulls, HG-TEQs were used to order the study sites for relative contamination. The Jonckheere test was performed using a custom-written FORTRAN program (B. Collins, Senior Statistician, Canadian Wildlife Service) using the algorithm provided by Gibbons (1971). This program determined probability values based on 5000 random permutations of the data set being analyzed. These probability values were very similar to those found by the large sample normal approximation for the Jonckheere test (Hollander and Wolfe 1973).

Before the Jonckheere test was performed, a preliminary ANOVA was used to determine whether the response variable was influenced by year or a year × site interaction for subsets of data that were collected at some sites during different years. If there was no statistically significant year or a year × site interaction effect ($P<0.05$), then data were pooled across years for the Jonckheere test.

If the Jonckheere tests did not show any statistically significant trends, then one-way ANOVA within years followed by Duncan's multiple range test was used to look for differences among sites. Even though the ANOVA did not reveal associations to contaminants, it did elucidate spatial differences in response variables. For ANOVA analyses, the heterophil/lymphocyte ratios were transformed (log_{10}) to satisfy assumptions of homogenous variances and normality (Zar 1984). This transformation also was used for the Jonckheere and correlation analyses for this variable.

One objective of this investigation was to determine whether immunological biomarkers were related to one another or to biochemical biomarkers. Associations between the various biomarkers were detected using Pearson's correlation analysis. Statistically significant correlations ($P<0.05$) were reported only if the absolute value of the correlation coefficient (r) was greater than 0.3.

Results

Growth

The most significant alteration of growth occurred in both herring gull and Caspian tern chicks in Saginaw Bay during 1992 (Fig. 4.2). At other sites, herring gull chicks gained 14-20 g/d between 3 and 4 weeks of age, and Caspian tern chicks gained 4-18 g/d. However, during 1992, severe body mass loss occurred (-11 g/d in herring gulls and -5 g/d in Caspian terns) in Saginaw Bay despite an apparently abundant food supply as assessed by the number of regurgitated food items and fresh pellets. High chick mortality and low fledging success accompanied this body mass loss. Considering only herring gulls from Saginaw Bay, during 1992, growth was 29 g/d lower than during 1993 and 19

g/d lower than during 1994. ($F_{2,85}$=20.8, P<0.0001). During 1992, growth in Saginaw Bay was 25-31g/d lower than at all other sites ($F_{4,62}$=7.69, P<0.0001). In Caspian tern chicks from Saginaw Bay and the North Channel, there was a significant year × site interaction during 1992-94 ($F_{2,180}$=3.23, P=0.042). During 1992, growth in Saginaw Bay was 11-23 g/d lower than at the other three sites ($F_{3,78}$=16.1, P<0.0001).

Organochlorine Concentrations

The range in organochlorine contaminants among sites was much greater for pooled herring gull eggs than for pooled Caspian tern eggs (Table 4.2). From the least to most contaminated herring gull colony, there was a 6.6× increase in total PCBs, a 6.7× increase in chicken bioassay TEQs, a 6.0× increase in HG-TEQs, and a 7.8× increase in DDE. In Caspian tern eggs, the increases were 1.8× for total PCBs, 4.2 for chicken bioassay TEQs, and 4.1× for DDE. Although minimum concentrations for PCBs and DDE were similar for the two species, maximum values were higher in herring gulls as compared to Caspian terns (3.6× for PCBs, 2.6× for chicken bioassay TEQs, and 2.1× for DDE). In herring gulls, total PCBs, chicken bioassay TEQs, HG-TEQs, and DDE gave the same rank order for contamination, but these orders were different in Caspian terns. Therefore, the Jonckheere test was identical for total PCBs, chicken bioassay TEQs, and DDE in herring gulls. In Caspian terns, these tests were different because some of the sites were different.

Functional Tests For Immunocompetence

In both herring gull and Caspian tern chicks, several measures of organochlorine contamination showed strong inverse relationships with T cell function as measured by the

PHA skin test (Table 4.3, Fig. 4.3). In herring gulls, preliminary ANOVA indicated no evidence that year or a year × site interaction influenced the stimulation index (P>0.45). Thus, data could be pooled across years. During 1992-94, there was very strong evidence that T-cell-mediated immunity decreased as total PCBs, chicken bioassay TEQs, HG-TEQs, and DDE increased (P=0.0002). The most contaminated sites were suppressed 35-45% compared to the least contaminated sites. Considering only 1992 data, there was still strong evidence for this inverse relationship (P=0.0009).

In Caspian tern chicks, preliminary ANOVA revealed marginal evidence that year affected the stimulation index ($F_{2,206}$=2.87, P=0.058), but this effect was much weaker than the site effect ($F_{1,206}$=31.1, P<0.0001). During 1992-94, there was very strong evidence that T-cell-mediated immunity decreased as total PCBs (P=0.0002), chicken bioassay TEQs (P=0.0002), and DDE (P=0.0002) exposure increased. During 1992 alone, there was very strong evidence for an inverse relationship between total PCBs (P=0.0004) and DDE (P=0.0008), but very weak evidence for such a relationship with chicken bioassay TEQs (P=0.14). The most contaminated sites were suppressed 30% compared to the least contaminated sites.

In both species, there was no evidence of contaminant-induced suppression of the primary total antibody (IgM + IgG) and IgG responses following immunization with SRBC (P>0.50; Table 4.3; Fig. 4.4). In Saginaw Bay herring gull chicks, the only site for which there were multiple years of data, there was marginal evidence that the total antibody response decreased from 1992-94 ($F_{2,84}$=2.50, P=0.088) but moderate evidence the IgG response decreased during this same time period ($F_{2,84}$=4.13, P=0.020). When combining 1992-94 data, there was very weak evidence for a difference among the four

sites in the total antibody ($F_{3,135}$=1.74, P=0.16) or IgG ($F_{3,135}$=1.53, P=0.21) responses. When considering only 1992 data, there was moderate evidence that site influenced the total antibody response ($F_{2,43}$=3.97, P=0.026) and strong evidence that site influenced the IgG response ($F_{2,43}$=7.06, P=0.0022). In Caspian tern chicks from Saginaw Bay and the North Channel, there was very strong evidence that year influenced the total antibody response ($F_{2,180}$=7.68, P=0.0006) and marginal evidence for a year × site interaction effect ($F_{1,180}$=2.57, P=0.079). There was strong evidence for a year × site interaction effect on the IgG response ($F_{2,179}$=5.24, P=0.0061). During 1992, there was strong evidence for differences among the three sites in the total antibody response ($F_{2,61}$=5.19, P=0.0083) and IgG response ($F_{2,61}$=5.76, P=0.0051). After examination of the differences in antibody titers among Caspian tern colonies, *a posteriori* Jonckheere tests were conducted to look for a positive relationships among contaminants and antibody titers. These tests provided strong evidence for positive associations between the total antibody response total PCBs, chicken bioassay TEQs, and DDE for data from 1992 alone and data from 1992-94 (P<0.02 for two-way tests).

General Immunological, Hematological, and Biochemical Biomarkers

In both species, several measures of organochlorine contamination showed strong inverse relationships with plasma retinol concentrations (Table 4.4, Fig. 4.5). In herring gull chicks from Saginaw Bay and the North Channel, the preliminary ANOVA provided little or no evidence that year ($F_{1,36}$=1.91, P=0.18) or a year × site interaction ($F_{1,36}$=0.057, P=0.81) influenced the plasma retinol concentration. During 1992-93, there

was moderate evidence that plasma retinol decreased as total PCBs, chicken bioassay TEQs, HG-TEQs, and DDE increased (P=0.014). In Caspian tern chicks from Saginaw Bay and the North Channel, the preliminary ANOVA revealed marginal evidence that a year × site interaction influenced plasma retinol ($F_{1,31}$=3.35, P=0.077), but little evidence for a year effect ($F_{1,31}$=2.16, P=0.15). During 1992-93, there was very strong evidence for a negative relationship between plasma retinol concentration and total PCBs (P=0.0002), chicken bioassay TEQs (P=0.0002), and DDE (P=0.0002).

There was little or no evidence that organochlorine contamination influenced plasma thyroxine concentrations (P>0.15; Table 4.4, Fig. 4.6). In herring gull chicks from Saginaw Bay and the North Channel, the preliminary ANOVA indicated strong evidence that a year × site interaction influenced plasma thyroxine ($F_{1,36}$=8.05, P=0.007). During 1992, there was very strong evidence that plasma thyroxine differed among sites ($F_{4,44}$=8.60, P<0.0001). In Caspian tern chicks, there was little or no evidence for a year × site interaction effect ($F_{1,33}$=0.16, P=0.69) and only marginal evidence for a year effect ($F_{1,33}$=3.15, P=0.085) on plasma thyroxine in Saginaw Bay and the North Channel. During 1992-93, there was very strong evidence that plasma thyroxine differed among the five sites ($F_{4,64}$=7.52, P<0.0001).

Differential white blood cell counts varied significantly among sites, but a relationship to organochlorine contaminants was evident only for Caspian tern chicks during 1992 (Tables 4.4 and 4.5). Only heterophils and lymphocytes were counted in numbers sufficient for statistical analysis. In herring gull chicks from Saginaw Bay and the North Channel, the preliminary ANOVA provided very strong evidence for a year × site interaction effect on percent heterophils ($F_{1,48}$=14.6, P=0.0004) and percent lymphocytes

($F_{1,48}$=13.2, P=0.0007). Considering only 1992 data, there was very strong evidence that site influenced both percent heterophils ($F_{4,42}$=5.64, P=0.001) and percent lymphocytes ($F_{4,42}$=6.26, P<0.0001). There was little or no evidence that organochlorine contaminants affected the heterophil/lymphocyte ratio of herring gulls during 1992 (P=0.26 for a two-way test).

In Caspian tern chicks from Saginaw Bay and the North Channel, the preliminary ANOVA provided very strong evidence for a year × site interaction effect on percent heterophils ($F_{1,53}$=48.6, P<0.0001) and on percent lymphocytes ($F_{1,53}$=29.6, P<0.0001). During 1992, there was very strong evidence that site influenced percent heterophils ($F_{4,41}$=13.7, P<0.0001) and percent lymphocytes ($F_{4,41}$=7.92, P<0.0001). During 1992, there was strong evidence that the heterophil/lymphocyte ratio increased with increasing total PCBs (P=0.0028 for a two-way test), chicken bioassay TEQs (P=0.0004 for a two-way test), and DDE (P=0.022 for a two-way test).

In both species there was a trend for an increase in packed red blood cell volume (PCV) as some measures of organochlorine contamination increased (Tables 4.4 and 4.5). In herring gull chicks from Saginaw Bay and the North Channel during 1992-93, there was strong evidence that year influenced PCV ($F_{1,78}$=7.40, P=0.008). During 1992, there was marginal evidence that PCV increased as total PCBs, chicken bioassay TEQs, HG-TEQs, and DDE increased (P=0.086 for a two-way test). This trend was strongly influenced by low PCVs at the Lake Winnipeg reference site. In Caspian tern chicks from Saginaw Bay and the North Channel, the preliminary ANOVA provided very strong evidence that a year × site interaction influenced PCV. During 1992, there was marginal evidence that PCV increased as total PCBs (P=0.085) and DDE (P=0.087) increased. This trend was strongly

influenced by low PCVs at the North Channel reference site. There was little or no evidence for such a relationship with chicken bioassay TEQs (P=0.55).

Relationships among Functional Tests and General Biomarkers

Pearson's correlation analysis was used to explore relationships among biomarkers (Table 4.6). The objective was to determine whether there were any associations between different immunological and hematological parameters and whether any biochemical biomarkers could serve as surrogates for immunological biomarkers.

In both herring gull and Caspian tern chicks, there was little evidence for any biologically significant relationships between T-cell-mediated and antibody-mediated immunity. In herring gulls, there were no statistically significant correlations. In Caspian terns during 1992, there was moderate evidence for a weak negative relationship between the PHA response and the total antibody titer (r= -0.31, P=0.015). During 1992-94, this relationship was even weaker (r= -0.16, P=0.017).

The PHA skin test response showed few relationships to any other biomarkers in either species. In herring gull chicks during 1992, the PHA response was positively correlated with plasma thyroxine (P=0.026). This relationship was much weaker during 1992-93 (P=0.085). In Caspian tern chicks, the PHA response was positively correlated with percent eosinophils during 1992-93 (P=0.004) and during 1992 alone (P=0.024).

The antibody responses were significantly correlated with white blood cell variables, but the nature of this relationship was different in the two species. In herring gull chicks during 1992-93, the heterophil/lymphocyte ratio was negatively correlated with the total antibody (P=0.002) and IgG (P<0.001) responses. In Caspian tern chicks during

1992-93, the heterophil/lymphocyte ratio was positively correlated with the total antibody ($P<0.001$) and IgG ($P=0.007$) responses.

Plasma retinol was positively correlated with the total antibody ($P=0.006$) and IgG ($P=0.033$) responses in herring gull chicks during 1992. This relationship was weaker when 1993 data were added to 1992 data ($P=0.093$ for total antibody, $P=0.24$ for IgG). Plasma retinol was not associated with antibody responses in Caspian tern chicks.

Several biomarkers were related to various measures of body size, body condition, or age. In herring gull chicks, plasma thyroxine was correlated with body mass ($P=0.013$), wing chord length ($P<0.001$), and body mass/ wing chord ratio ($P=0.007$) during 1992. Weaker relationships were observed for 1992-93 data ($P=0.051$ for body mass; $P=0.002$ for wing chord; $P=0.037$ for body mass/wing chord ratio). During 1992, plasma retinol was positively correlated with body mass ($P=0.013$), wing chord length ($P<0.001$) and body mass/wing chord ratio ($P=0.007$) in herring gull chicks. Weaker relationships were observed for 1992-93 data ($P=0.0029$ for body mass; $P=0.019$ for wing chord length; $P=0.16$ for body mass/wing chord ratio). Neither plasma thyroxine nor retinol were associated with body size in Caspian tern chicks. In herring gull chicks, the heterophil/lymphocyte ratio was negatively correlated with body mass ($P=0.003$) and wing chord length ($P=0.007$) during 1992. Weaker relationships were observed during 1992-93 ($P=0.004$ for body mass; $P=0.027$ for wing chord). PCV was positively correlated with wing chord ($P<0.001$) in Caspian tern chicks during 1992-4. Percent eosinophils were positively correlated with daily body mass gain in Caspian tern chicks during 1992 ($P=0.035$) during 1992-93 ($P<0.001$).

Discussion

The results of this field investigation demonstrated contaminant-associated suppression of T-cell-mediated, but not antibody-mediated, immune function in chicks of two species of colonial waterbirds in the Great Lakes. These associations with T cell function were consistent with the contaminant-associated thymic atrophy found in herring gull chicks from the Great Lakes (Chapter 3). The PHA skin test was an extremely effective and sensitive biomarker for assessing T cell function in wild birds. The within site variation was low enough to allow statistical differentiation among sites, especially when the sample sizes within sites were greater than 20 birds. At sites where the test was replicated for two or three years, the response was consistent among years. It was not influenced by year or year × site interaction effects. For both species in Saginaw Bay, results were similar during the year of severe body mass loss and mortality (1992) as compared to the following two years of normal growth and fledging success. Lochmiller *et al.* (1993) found that the PHA skin test was much more sensitive to the immunosuppressive effects of a low protein diet in northern bobwhites (*Colinus virginianus*) as compared to *in vitro* proliferation of T lymphocytes in response to PHA. The *in vitro* T lymphocyte proliferation assay measures only very early events in the T cell response that are involved with cell division. In contrast, the *in vivo* skin test incorporates a number of events in the T cell response, including cell proliferation, differentiation, and cytokine production (Stadecker *et al.* 1977, Lochmiller *et al.* 1993).

The PHA test has been used as a test for T cell function in laboratory experiments and in human and veterinary clinical medicine. When T lymphocyte function was obliterated using irradiation or immunosuppressive drugs in laboratory chickens and ducks, the PHA response was reduced 50-60% as compared to controls (Edelman *et al.* 1986,

Schrank *et al.* 1990, Grasman and Scanlon 1995). Hence, the 30-45% suppression of this response in herring gulls and Caspian terns at highly contaminated sites in the Great Lakes represents a biologically significant impact on the immune system. This 30-45% suppression may represent the maximal level of suppression in these species. In both species, the occurrence of severe body mass loss in Saginaw Bay during 1992 did not result in additional suppression of the response as compared to other years.

Suppression of the PHA skin response occurred over a much narrower exposure range in Caspian tern chicks than in herring gull chicks. In Caspian tern chicks, the response was suppressed approximately 30% over an exposure range from 4.3 to 7.7 μg/g in eggs. In contrast, the 30-45% suppression in herring gull chicks occurred over an exposure range from 4.2 to 27.5 μg/g in eggs. The sensitivity of Caspian tern chicks to the immunosuppressive effects of organochlorines is not surprising considering their sensitivity to the reproductive effects of these pollutants. The lower contaminant concentrations in Caspian tern eggs as compared to herring gull eggs are probably related to the migratory habits of the terns. The terns migrate to Central and South America for six months of the year, where they presumably eat a less-contaminated food supply when inhabiting ocean beaches. When Caspian terns return to breed at highly contaminated Great Lakes sites such as Saginaw Bay, the body burden of contaminants in female terns increases throughout the breeding season. Second clutch eggs have higher organochlorine concentrations and lower rates of hatching than first clutch eggs (Ludwig *et al.* 1993). In contrast, herring gulls are year-round residents of the Great Lakes, so they are chronically exposed to high concentrations of organochlorines.

Because most organochlorines tend to biomagnify up the food web, their concentrations tend to be highly co-correlated. Hence, it is difficult to determine which

organochlorine contaminants are responsible for T-cell-mediated immunosuppression in Great Lakes colonial waterbirds. In herring gull eggs, gradients in PCBs and DDE all occurred in the same rank order. However, PCBs occurred at much higher concentrations than DDE. Chick-bioassay-derived TEQs are a measure of TCDD-like activity of PCBs, PCDDs, and PCDFs. In Caspian tern chicks, the PHA skin response decreased as total PCBs, chicken bioassay TEQs, and DDE increased. However, the strongest relationship was with PCBs. As in herring gulls, PCBs had the highest concentrations of any organochlorines in Caspian tern eggs. While PCBs were probably the major cause of immunosuppression in both species, the effects of and interactions with other organochlorine contaminants cannot be ruled out.

The SRBC hemagglutination test was a good biomarker for measuring the function of antibody-mediated immunity in wild colonial waterbirds. The within site variability was low compared to among site variability, allowing statistical differentiation among sites. The IgG titers usually gave smaller P-values in ANOVA analyses as compared to the total antibody titers, suggesting that IgG is a more sensitive biomarker than the total antibody titer. A disadvantage of this assay is the need to make two visits to each colony six (5-7) days apart. The primary antibody response peaks in colonial waterbirds approximately six days after immunization. Poor weather and other logistical problems can make it difficult to return to a colony during this narrow time window, especially if the immunological tests are being conducted simultaneously at several sites.

At sites where the hemagglutination test was replicated for three years, the response often changed from year to year. Time trends in antibody titers were confounded by a change in the individual sheep that served as a source of SRBC. Between the 1993 and 1994 field seasons, the sheep that had been used previously died, so a sheep of similar age

from the same flock was substituted for 1994. In Saginaw Bay herring gull chicks, there was a nonsignificant decrease in total antibody titer over the three years. The IgG response decreased significantly from 1992 to 1993 but not from 1993 to 1994. In Caspian tern chicks from Saginaw Bay and the North Channel, the total antibody response dropped from 1992 to 1993 and again from 1993 to 1994. The IgG response showed a significant year × site interaction. In the North Channel, the IgG titers dropped significantly from 1992 to 1994, although the 1993 titers were not significantly different than the early or later years. In Saginaw Bay, IgG titers dropped significantly from 1992 to 1993, but not from 1993 to 1994. In both herring gull and Caspian tern chicks from Saginaw Bay, IgG titers dropped between 1992 and 1993 while the same source of SRBCs was used. This drop in IgG coincided with a doubling in total PCBs and DDE in herring gull eggs from 1992 to 1993 (D.V. Weseloh, Canadian Wildlife Service, pers. commun.). During this same time period, the zebra mussel (*Dreissenna polymorpha*) populations in Saginaw Bay increased greatly, possibly influencing contaminant cycling. Contaminant data for herring gull eggs from 1994 are not yet available.

Making comparisons among sites, there was no evidence for contaminant-induced suppression of the antibody responses. However, the magnitude of differences in total antibody and IgG titers among sites was biologically significant. Other factors such as genetics, nutrition, stress, and weather might have influenced antibody titers. *A posteriori* Jonckheere tests provided strong evidence for higher antibody titers in Caspian terns from more contaminated sites. Such trends suggest contaminant-associated deregulation of antibody-mediated immunity, but additional data should be gathered to investigate these patterns.

The antibody responses displayed different relationships to differential white blood cell counts in the two species. In herring gulls, the antibody responses increased as the relative number of heterophils to lymphocytes decreased. Thus, antibody-mediated immunity increased as the relative number of lymphocytes, the antibody-producing cells, increased. The heterophil to lymphocyte ratio accounted for 25-31% of the variation in the antibody responses in herring gull chicks. Conversely, in Caspian terns the antibody responses increased as the relative number of heterophils to lymphocytes increased. In this species the correlation explained only 12-17% of the variability in the data. Data for total white blood cell numbers, especially total lymphocytes, might clarify the antibody relationships to differential counts. However, total white blood cell counts can be difficult to determine under field conditions.

The suppression of T-cell-mediated immunity but not antibody-mediated immunity in this field study is consistent with laboratory studies on the effects of chronic and (or) perinatal exposure to PCBs and TCDD. These studies have documented consistent effects of PCBs and TCDD on T-cell-mediated immunity, including thymic atrophy and suppression of various T cell functions. In particular, T cell development is affected at various stages, including the prothymocyte (Fine *et al.* 1990) and thymocyte periods. The transition from CD4-CD8+ thymocytes to CD4+CD8+ thymocytes is inhibited (Blaylock *et al.* 1992), later resulting in a loss of helper T cell function. Similar reductions in helper T lymphocyte numbers have been observed in humans who have been exposed perinatally to PCBs, PCDDs, and PCDFs in arctic Quebec (Dewailly *et al.* 1993) and to TCDD in Times Beach, Missouri (Smoger *et al.* 1993). At Times Beach these effects persisted at least 10 years after the time period of highest exposure. In experiments with laboratory rodents, PCBs most consistently suppress antibody-mediated immunity at high acute doses rather

than at the chronic developmental exposures observed in this field study. At these high acute doses, PCBs and TCDD appear to act directly on B lymphocytes, preventing their differentiation into antibody-producing cells (Luster *et al.* 1988a).

Even though the anti-SRBC antibody response depends on helper T lymphocytes, the suppression of T-cell-mediated immunity but not antibody-mediated immunity in this field study is consistent with current immunological theory. The contaminant-associated suppression of the PHA skin response may reflect suppression of a subset of helper T lymphocytes that boost inflammatory responses but not antibody-mediated responses. In mice, T_H1 cells promote inflammatory, cytotoxic responses while T_H2 cells promote antibody responses. Suppression of the PHA skin response in herring gulls and Caspian terns suggests suppression of T_H1-like cells that would not necessarily enhance antibody responses. In laboratory animals, HAHs have been shown to suppress such inflammatory (delayed-type hypersensitivity) and cytotoxic responses (Vos and Van Driel-Grootenhuis 1972; Faith and Moore 1977; Sharma *et al.* 1978; Hinsdill *et al.* 1980; Thomas and Hinsdill 1980; Clark *et al.* 1981, 1983; Nagarkatti *et al.* 1984). In many cases, these cell-mediated responses are suppressed without any effects on production antibodies that depend on helper T cell (T_H1) activity (Faith and Moore 1977, Thomas and Hinsdill 1980, Clark *et al.* 1983).

Two recent studies have demonstrated contaminant-induced immunosuppression in marine mammals chronically exposed to organochlorines, including PCBs. Ross *et al.* (1995) found reduced *in vivo* delayed-type hypersensitivity (T-cell-mediated) and antibody responses to ovalbumin in harbor seals fed organochlorine-contaminated herring from the Baltic Sea. This same group found increased numbers of polymorphonuclear granulocytes, reduced NK cell activity, and reduced mitogen-induced proliferative T cell

responses in the organochlorine-exposed seals (De Swart *et al.* 1994). Mitogen-induced proliferative B cell responses were not affected. PCBs were the most prevalent contaminants found in the blubber of seals fed these fish. This contaminant-associated immunosuppression might have contributed to the 1988 phocine distemper virus epizootic in European harbor seals. Lahvis *et al.* (1995) found that the mitogen-induced proliferative T cell responses were inversely correlated with blood PCB and DDE concentrations in male bottlenose dolphins. B cell proliferation showed no relationships to contaminants.

The biochemical biomarkers (plasma retinol and thyroxine) were not consistently related to immunological function in individual birds. Retinol (vitamin A) is essential for immunocompetence. Furthermore, laboratory and field studies have demonstrated that organochlorines can disrupt vitamin A homeostasis. In this study, plasma retinol was strongly correlated with antibody responses in herring gull chicks during 1992 but less so when the data for 1993 were added. Retinol did not appear to influence immune function in Caspian tern chicks, although retinol concentrations were one to two orders of magnitude lower in terns as compared to gulls. Apparently, physiological regulation and (or) dietary intake of vitamin A differs greatly in these two species. Nonetheless, in both species plasma retinol decreased as PCB contamination increased, exhibiting a similar relationship to contamination as the PHA skin test. In herring gull chicks, this negative relationship was strongly influenced by the North Channel site, which had much higher retinol values than the Lake Winnipeg site, which was the other reference colony. Thus, although individual variability in retinol and the PHA skin response was great, on a site basis plasma retinol paralleled T-cell-mediated immunity.

Plasma thyroxine showed a strong relationship to the PHA skin response in herring gull chicks during 1992, although this relationship was weaker when 1993 data was added.

However, there was a strong relationship when comparing site means. There was little or no evidence for such a relationship in Caspian terns either on an individual bird or site basis. Although thyroxine and thyroid stimulating hormone often influence immune function in laboratory studies, there was little evidence that plasma thyroxine was a good surrogate biomarker for immune function in this field study.

Although the measures of immunological function were not confounded by growth or development, several general biomarkers were related to body size or condition. Larger herring gull chicks tended to have higher plasma thyroxine, plasma retinol, and PCVs than smaller chicks. Larger chicks also tended to have proportionally more lymphocytes than heterophils. In Caspian tern chicks, only PCV and percent eosinophils were positively correlated with body size or condition. These results suggest that many general biomarkers are related to growth in young birds, complicating their use as biomarkers for contaminant effects and exposure. As birds grow, the length of their exposure to pollutants also increases, so these associations could be influenced by contaminant exposure as well as normal growth patterns. These relationships were evident even though the only chicks selected for measurement were those that fit body mass and wing chord criteria for approximately 21 days of age.

The results of this field investigation suggest that most of the general biomarkers and immune function biomarkers used here can be important indices for assessing the health of free-living birds. However, few of the general biomarkers were clearly related to immunological function, suggesting that immune function must be measured directly, i.e., none of the general or structural biomarkers measured here provides a surrogate measure of T-cell-mediated or antibody-mediated immune function. However, general biomarkers such as plasma retinol are influenced by exposure to organochlorines such as PCBs.

Alterations in variables such as retinol and white blood cell counts suggest biologically significant differences in physiology and health among various Great Lakes colonies.

Conclusions

This study clearly demonstrated contaminant-associated suppression of T-cell-mediated immunity and plasma retinol levels in two species of fish-eating birds from the Great Lakes. Contaminant-associated impairment of T cell function was consistent with contaminant-associated thymic atrophy in herring gull chicks. The identity of the particular organochlorine(s) responsible for such suppression could not be determined since exposure to different organochlorines often is correlated due to similarities in environmental chemistry and metabolism. However, PCBs are the most likely cause for these effects because PCB concentrations were by far the highest of any immunosuppressive organochlorines in both species. Additional research is needed to determine the relationship between suppression of functional assays and increased susceptibility to infectious diseases. Such research will be important for determining the consequences of this T-cell-mediated immunosuppression on individual survival and population dynamics (Ludwig *et al.* 1993, Mora *et al.* 1993).

Table 4.1. Sampling design for immune function study in fish-eating birds of the Great Lakes during 1992-94.

Species	Year	Colony	Variable				
			PHA Test	SRBC Test	WBC Counts	Retinol	Thyroxine
Herring Gull	1992	Lake Winnipeg	X		X	X	X
		North Channel	X		X	X	X
		Hamilton Harbour	X	X	X	X	X
		W. Erie	X	X	X	X	X
		Saginaw Bay	X	X	X	X	X
	1993	North Channel	X	X	X	X	X
		Saginaw Bay	X	X	X	X	X
	1994	Saginaw Bay	X	X			
Caspian Tern	1992	North Channel	X	X	X	X	X
		Upper Green Bay	X		X	X	X
		N. Lake Michigan	X	X	X	X	X
		Saginaw Bay	X	X	X	X	X
		E. Lake Ontario	X		X	X	X
	1993	North Channel	X	X	X	X	X
		Sagainaw Bay	X	X	X	X	X
	1994	North Channel	X	X			
		Saginaw Bay	X	X			

Table 4.2. Organochlorine contaminants in pooled samples of herring gull and Caspian tern eggs from the Great Lakes and Lake Winnipeg during 1992.

Species/ Colony	Island/Location	Lake	Concentration (wet weight)							
			ΣPCBs (μg/g)	C- TEQs (ng/g)	HG-TEQs (pg/g)	p,p′-DDE (μg/g)	Dieldrin (μg/g)	Mirex (μg/g)	HCB (μg/g)	Heptachlor Epoxide (μg/g)
Herring Gull										
Lake Winnipeg	Pony Island	Winnipeg	4.17	2.60	71	1.00	0.11	0.03	0.03	0.06
North Channel	Bird/Anchor Islands	Huron	6.67	3.18	181	4.03	0.31	0.12	0.04	0.12
Hamilton Harbour	Hamilton Harbour	Ontario	14.18	9.22	240	.21	0.07	0.60	0.04	0.04
W. Erie	Monroe	Erie	21.39	13.48	257	5.79	0.20	0.08	0.04	0.10
Saginaw Bay	Little Charity Island	Huron	27.45	17.53	421	7.78	0.19	0.06	0.05	0.11
Caspian Tern										
North Channel	Elm Island	Huron	4.31	3.29		0.93	0.09	0.02	0.01	0.03
Upper Green Bay	Gravelly Island	Michigan	5.88	1.62		2.27	0.16	0.02	0.01	0.06
N. Lake Michigan	High Island	Michigan	6.57	4.38		3.46	0.16	0.02	0.01	0.06
Saginaw Bay	Confined Disposal Facility	Huron	7.54	6.84		3.12	0.02	0.03	0.01	0.02
E. Lake Ontario	Pigeon Island	Ontario	7.73	3.49		3.78	0.004	0.60	0.01	0.02

Table 4.3. Effects of contaminants on immune function in herring gull and Caspian tern chicks from the Great Lakes and Lake Winnipeg during 1992-94. Organochlorine contaminants were measured in pooled egg samples.

Dependent Variable	Independent Variable	Species	Year(s)	Predicted Trend	Actual Trend	Test for Ordered Alternatives	
						Jonckheere Statistic	P-Value
PHA Skin Test	PCBs Chicken TEQs HG-TEQs DDE[1]	Herring Gull	1992-94	-	-	**3327**	**0.0002**
			1992	-	-	**603**	**0.0009**
	PCBs	Caspian Tern	1992-94	-	-	**10,211**	**0.0002**
			1992	-	-	**1821**	**0.0004**
	Chicken TEQs	Caspian Tern	1992-94	-	-	**8543**	**0.0002**
			1992	-	-	387	0.14
	DDE	Caspian Tern	1992-94	-	-	**7073**	**0.0002**
			1992	-	-	**1589**	**0.0008**
Total Antibody Response	PCBs Chicken TEQs HG-TEQs DDE[1]	Herring Gull	1992-94	-	+	-463	0.56
			1992	-	+	-99	0.73
	PCBs Chicken TEQs[1]	Caspian Tern	1992-94	-	+	-3546	0.99
			1992	-	+	-602	1.0
	DDE	Caspian Tern	1992-94	-	+	-5698	1.0
			1992	-	+	-582	1.0

[1] The rank orders of sites for were identical for the designated variables, and thus the Jonckheere test was identical.

Table 4.4. Effects of contaminants on general immunological and hematological biomarkers in herring gull and Caspian tern chicks from the Great Lakes and Lake Winnipeg during 1992-94. Organochlorine contaminants were measured in pooled egg samples.

Dependent Variable	Independent Variable	Species	Year(s)	Predicted Trend	Actual Trend	Test for Ordered Alternatives	
						Jonckheere Statistic	P-Value
Plasma Retinol	PCBs Chicken TEQs HG-TEQs DDE[1]	Herring Gull	1992-94	-	-	**422**	**0.014**
	PCBs	Caspian Tern	1992-94	-	-	**310**	**0.0002**
	Chicken TEQs	Caspian Tern	1992-94	-	-	**330**	**0.0002**
	DDE	Caspian Tern	1992-94	-	-	**174**	**0.0002**
Plasma Thyroxine	PCBs Chicken TEQs HG-TEQs DDE[1]	Herring Gull	1992-94	-	-	174	0.17
	PCBs	Caspian Tern	1992-94	-	+	-124	0.74
	Chicken TEQs	Caspian Tern	1992-94	-	+	-350	0.97
	DDE	Caspian Tern	1992-94	-	+	-68	0.65

[1] The rank orders of sites for were identical for the designated variables, and thus the Jonckheere test was identical.

Table 4.4 (continued).

Dependent Variable	Independent Variable	Species	Year(s)	Predicted Trend	Actual Trend	Test for Ordered Alternatives	
						Jonckheere Statistic	P-Value
Heterophil/ Lymphocyte Ratio (Log_{10})	PCBs Chicken TEQs HG-TEQs DDE	Herring Gull	1992-94	+/-	+	195	0.40
			1992	+/-	+	122	0.26
	PCBs	Caspian Tern	1992-94	+/-	-	31	0.88
			1992	+/-	+	**307**	**0.0028**
	Chicken TEQs	Caspian Tern	1992-94	+/-	+	69	0.78
			1992	+/-	+	**371**	**0.0004**
	DDE	Caspian Tern	1992-94	+/-	+	127	0.62
			1992	+/-	+	**239**	**0.022**
PCV	PCBs Chicken TEQs HG-TEQs DDE	Herring Gull	1992-94	+/-	+	363	0.086
			1992	+/-	+	**263**	**0.028**
	PCBs	Caspian Tern	1992-94	+/-	+	**3437**	**0.0004**
			1992	+/-	+	95	0.085
	Chicken TEQs	Caspian Tern	1992-94	+/-	+	-141	0.054
			1992	+/-	+	-419	0.55
	DDE	Caspian Tern	1992-94	+/-	+	**4787**	**0.0004**
			1992	+/-	+	119	0.087

[1] The rank orders of sites for were identical for the designated variables, and thus the Jonckheere test was identical.

Table 4.5. Means (± one standard error) of hematological variables in herring gull and Caspian tern chicks from the Great Lakes and Lake Winnipeg during 1992-93. Numbers in parentheses indicate sample sizes.

Species/ Colony	Year	Differential WBC Counts (%)					Heterophil/ Lymphocyte Ratio	PCV (%)
		Heterophils	Lymphocytes	Monocytes	Basophils	Eosinophils		
Herring Gull								
Lake Winnipeg	1992	59.7±5.1 (10)	34.2±4.8 (10)	4.6±0.7 (10)	1.4±0.5 (10)	0.1±0.1 (10)	2.39±0.57 (10)	25.2±0.8 (10)
North Channel	1992	34.5±4.4 (10)	62.6±4.5 (10)	2.8±0.6 (10)	0.05±0.05 (10)	0.0±0.0 (10)	0.63±0.13 (10)	28.9±1.7 (11)
	1993	49.9±3.5 (13)	45.2±3.4 (13)	4.3±0.7 (13)	0.7±0.3 (13)	0.0±0.0 (13)	1.26±0.19 (13)	30.4±0.8 (13)
Hamilton Harbour	1992	44.2±5.0 (8)	50.4±5.0 (8)	3.8±1.2 (8)	1.6±0.4 (8)	0.0±0.0 (8)	1.01±0.20 (8)	31.0±0.9 (5)
W. Erie	1992	54.6±5.2 (10)	41.2±5.1 (10)	3.8±1.0 (10)	0.6±0.4 (10)	0.0±0.0 (10)	1.65±0.32 (10)	32.8±0.4 (27)
Saginaw Bay	1992	61.4±4.2 (9)	35.3±4.5 (9)	3.3±0.6 (9)	0.0±0.0 (9)	0.0±0.0 (9)	2.16±0.46 (9)	29.1±0.8 (14)
	1993	50.2±2.2 (20)	43.1±2.2 (20)	6.0±0.8 (20)	0.6±0.1 (20)	0.1±0.1 (20)	1.28±0.12 (20)	32.0±0.3 (44)
	1994							30.2±0.4 (38)

Table 4.5 (continued).

Species/ Colony	Year	Differential WBC Counts (%)					Heterophil/ Lymphocyte Ratio	PCV (%)
		Heterophils	Lymphocytes	Monocytes	Basophils	Eosinophils		
Caspian Tern								
North Channel	1992	38.2±2.3 (10)	45.4±3.0 (10)	0.6±0.2 (10)	3.2±0.8 (10)	12.7±2.5 (10)	0.90±0.10 (10)	30.8±0.4 (30)
	1993	58.3±2.1 (20)	30.8±2.0 (20)	1.0±0.3 (20)	0.08±0.04 (20)	9.7±1.1 (20)	2.18±0.25 (20)	27.4±0.3 (31)
	1994							30.3±0.4 (20)
Upper Green Bay	1992	52.0±3.1 (10)	36.0±2.6 (10)	1.1±0.3 (10)	0.4±0.2 (10)	10.4±1.4 (10)	1.59±0.23 (10)	32.2±0.3 (40)
N. Lake Michigan	1992	56.2±3.4 (10)	28.0±2.7 (10)	0.2±0.2 (10)	0.9±0.4 (10)	14.6±1.9 (10)	2.27±0.32 (10)	32.7±0.5 (31)
Saginaw Bay	1992	71.8±4.8 (7)	22.4±4.6 (7)	0.07±0.07 (7)	0.3±0.15 (7)	5.5±1.1 (7)	4.62±1.29 (7)	32.3±1.3 (7)
	1993	47.1±3.0 (20)	43.0±2.9 (20)	1.9±0.4 (20)	0.6±0.2 (20)	7.5±0.7 (20)	1.30±0.18 (20)	31.2±0.3 (43)
	1994							31.8±0.4 (31)
E. Lake Ontario	1992	50.4±2.0 (9)	36.4±2.5 (9)	0.5±0.2 (9)	3.1±0.7 (9)	9.6±1.3 (9)	1.47±0.16 (9)	31.5±0.5 (20)

Table 4.6. Pearson's correlation analysis exploring relationships among biomarkers in herring gull and Caspian tern chicks from the Great Lakes and Lake Winnipeg during 1992-94.

Relationship	Species	Year(s)	Variables		r	N	P
Among Measures of Immune Function	Caspian Tern	92	PHA	Antibody	-0.31	63	0.015
		92-94			-0.16	214	0.017
Among Immune Function and other Biomarkers	Herring Gull	92	PHA	Thyroxine	0.32	47	0.026
		92-93			0.21	67	0.085
	Caspian Tern	92	PHA	%Eosinophils	0.33	46	0.024
		92-93			0.31	85	0.004
	Herring Gull	92-93	Antibody	H/L Ratio[1]	-0.50	37	0.002
			IgG	H/L Ratio	-0.56	37	<0.001
	Caspian Tern	92-93	Antibody	H/L Ratio	0.41	58	<0.001
			IgG	H/L Ratio	0.35	58	0.007
	Herring Gull	92	Antibody	Retinol	0.68	15	0.006
		92-93			0.31	31	0.09
		92	IgG	Retinol	0.55	15	0.033
		92-93			0.22	31	0.24
Among Body Size and Biomarkers	Herring Gull	92	Thyroxine	Body Mass	0.36	48	0.013
		92-93			0.23	69	0.051
		92	Thyroxine	Wing Chord	0.45	48	<0.001
		92-93			0.37	69	0.002
		92	Retinol	Body Mass	0.36	48	0.013
		92-93			0.26	68	0.03
		92	Retinol	Wing Chord	0.45	48	<0.001
		92-93			0.28	68	0.019
		92	H/L Ratio	Body Mass	-0.43	47	0.003
		92-93			-0.32	80	0.004
		92	H/L Ratio	Wing Chord	-0.39	47	0.007
		92-93			-0.25	80	0.027
	Caspian Tern	92-94	PCV	Wing Chord	0.33	253	<0.001
		92	PCV	Daily Growth	0.29	29	0.035
		92-93			0.47	64	<0.001

[1] Heterophil/Lymphocyte Ratio

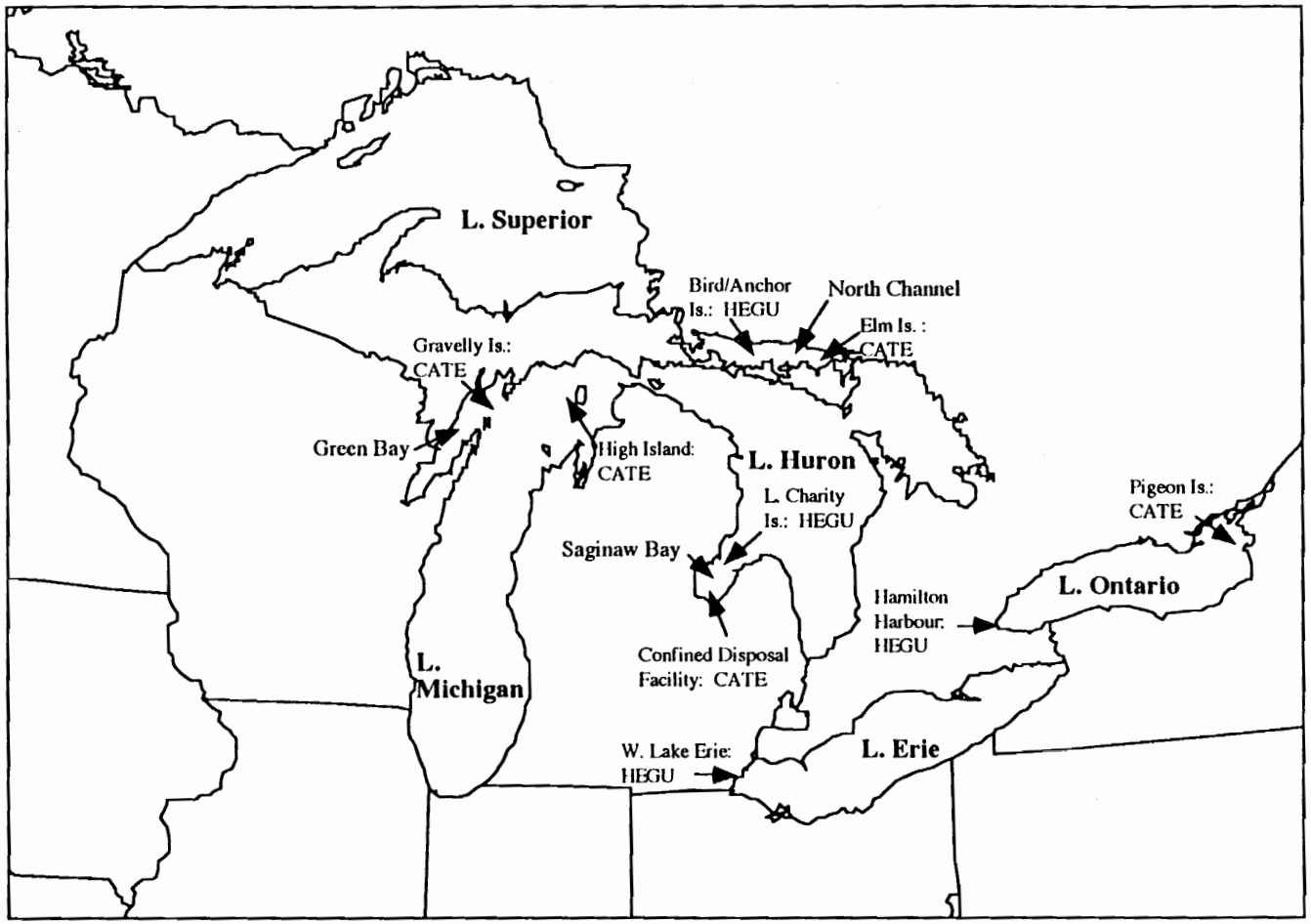

Fig. 4.1. Great Lakes sampling sites for immune function tests in herring gull (HEGU) and Caspian tern (CATE) chicks. Herring gull chicks were also sampled on Pony Island in northern endof Lake Winnipeg, Manitoba, Canada. Birds were sampled at all sites during 1992. Birds were also sampled at Bird/Anchor Islands Islands during 1993 and at Elm Is., L. Charity Is., and the Confined Disposal Facility during 1993 and 1994.

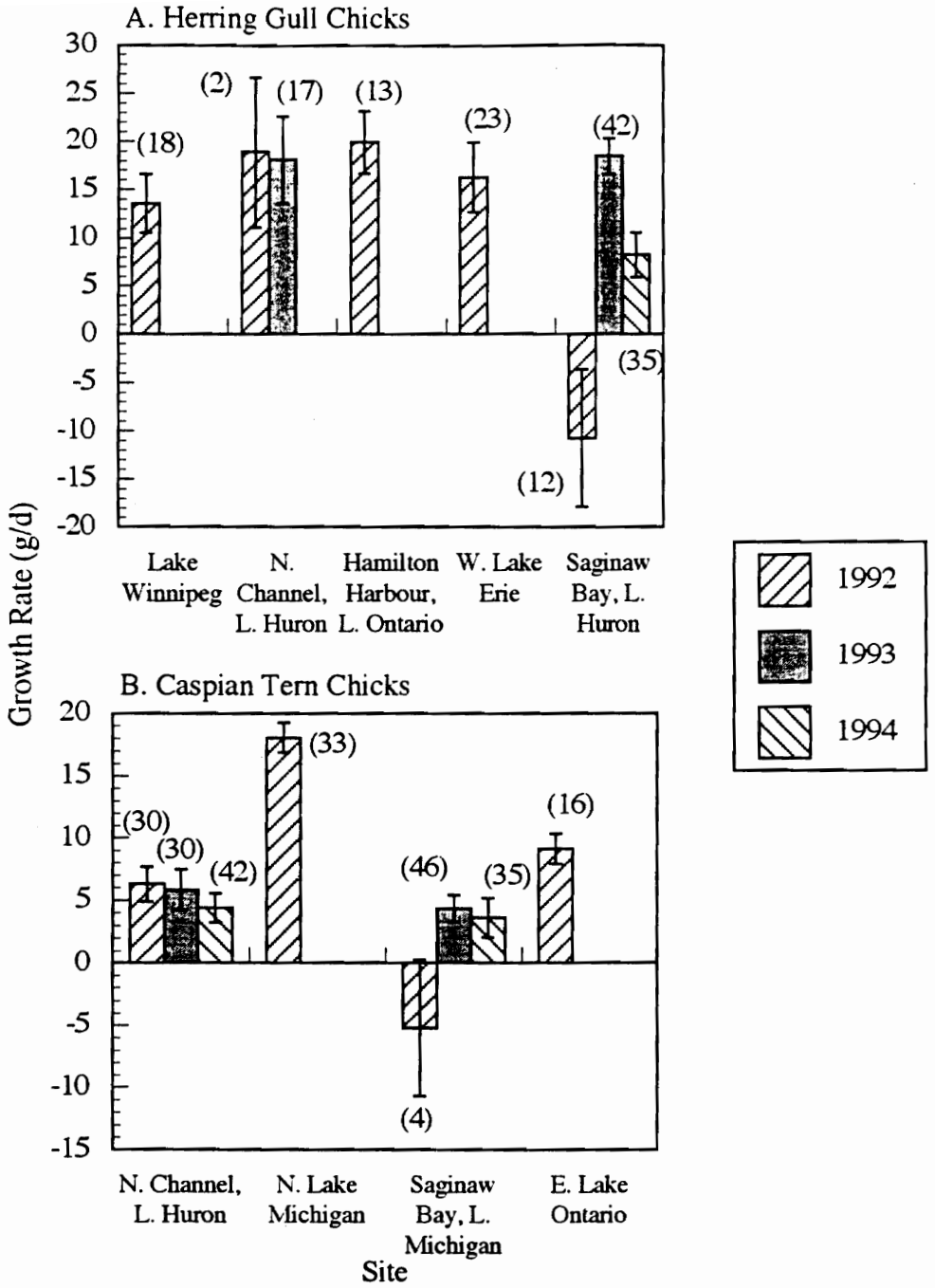

Fig. 4.2. Mean daily growth in 3-4 week old herring gull (A) and Caspian tern (B) chicks from the Great Lakes and Lakes Winnipeg during 1992-94. Error bars indicate one standard error of the mean. Numbers in parentheses indicate sample sizes.

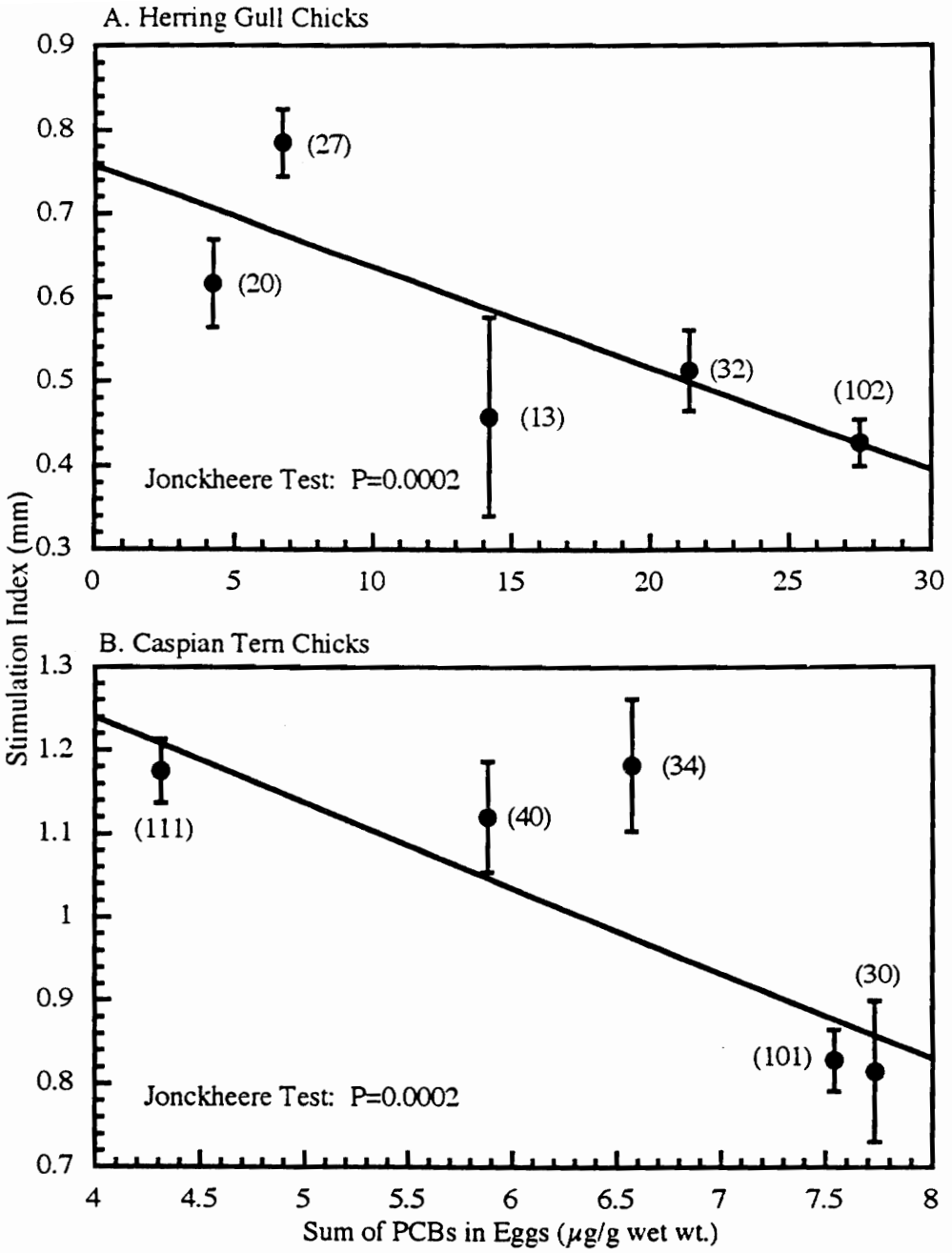

Fig. 4.3. Relationship between T-cell-mediated immunity (PHA skin test) and PCB contamination in herring gull (A) and Caspian tern (B) chicks from the Great Lakes and Lakes Winnipeg during 1992-94. Closed circles indicate mean response for each site. Error bars indicate one standard error of the mean. Numbers in parentheses indicate sample sizes.

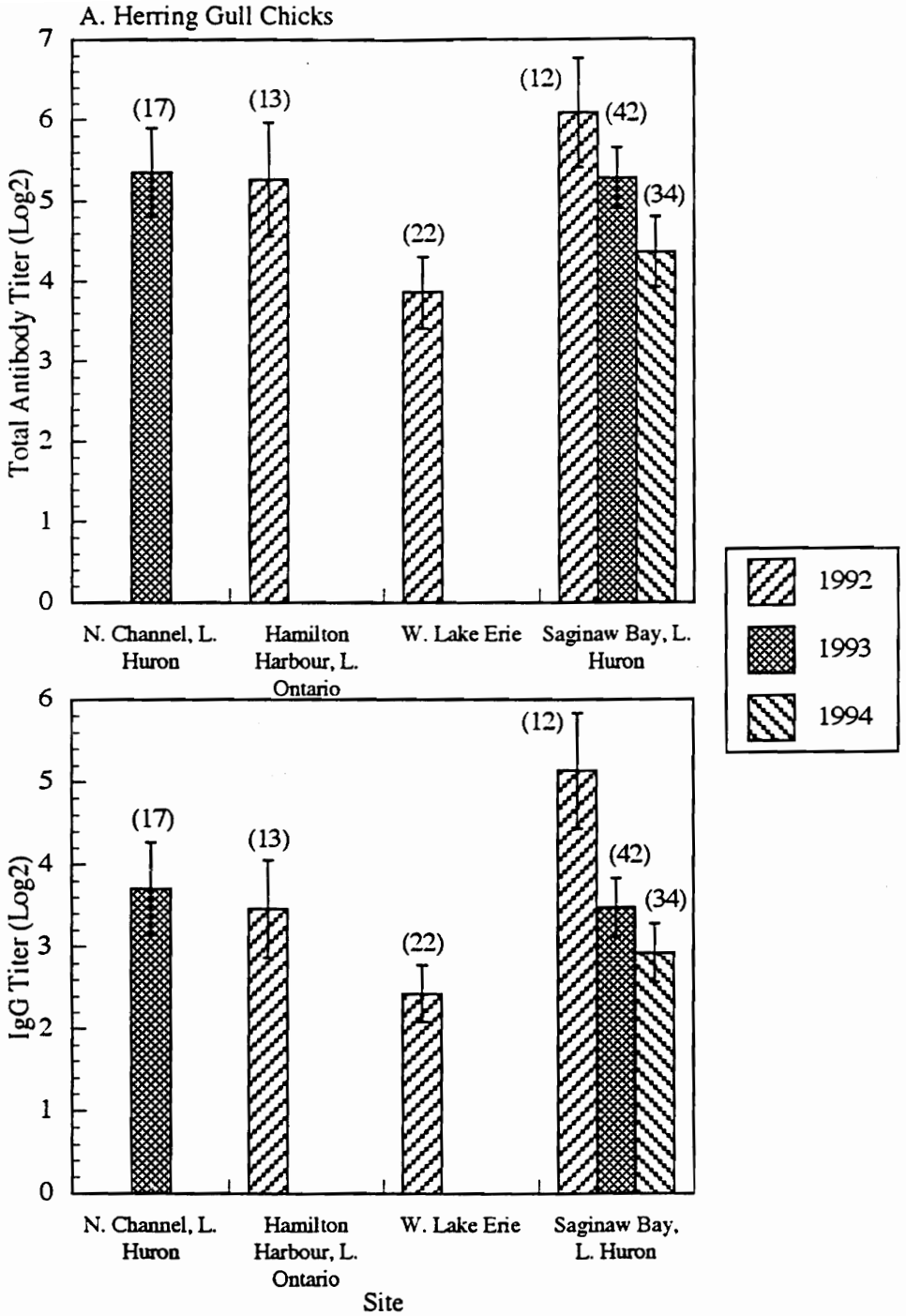

Fig. 4.4. Mean primary antibody responses 5-7 days after SRBC immunization in herring gull (A) and Caspian tern (B) chicks from the Great Lakes during 1992-94. Error bars indicate one standard error of the mean. Numbers in parentheses indicate sample sizes.

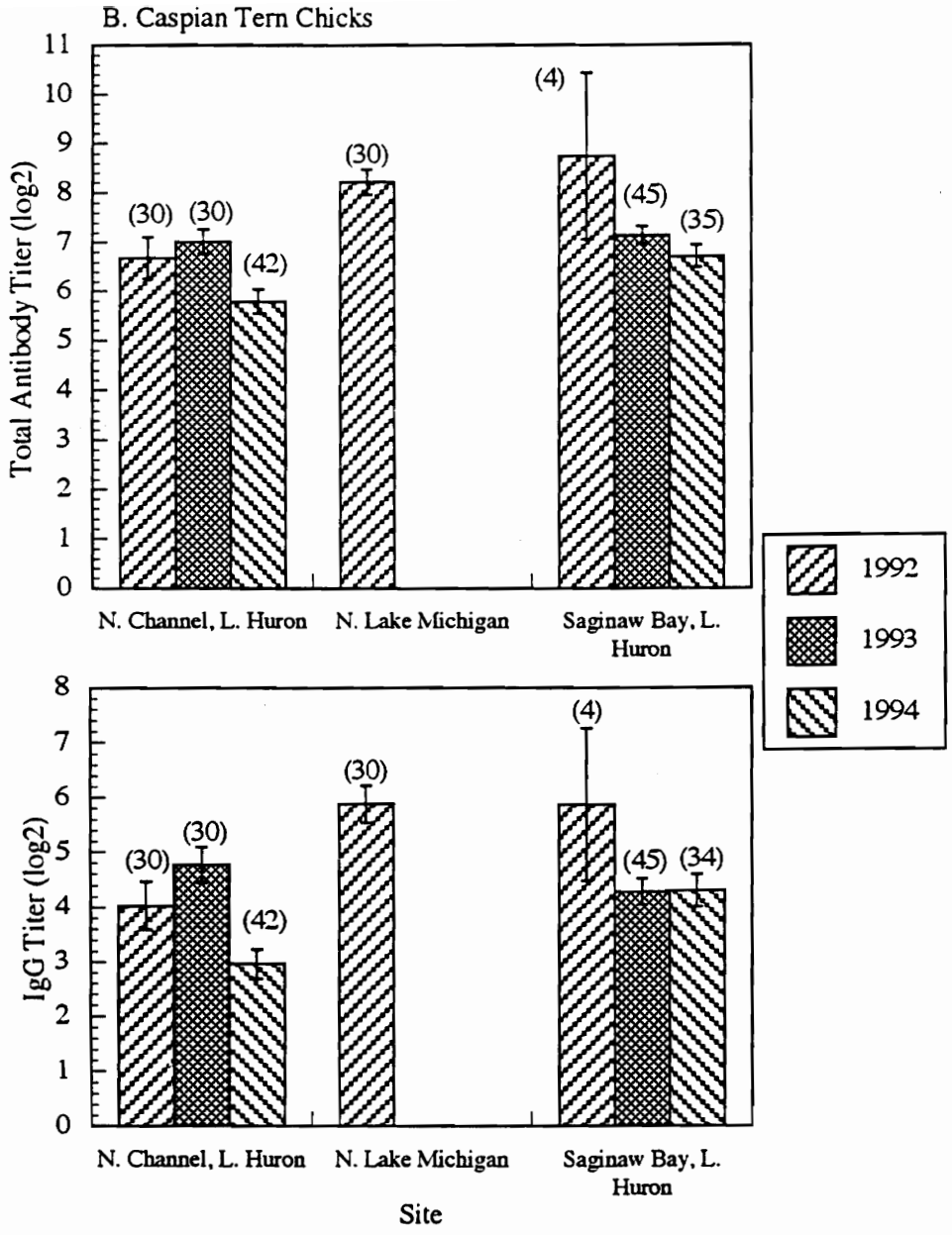

Fig. 4.4. (continued).

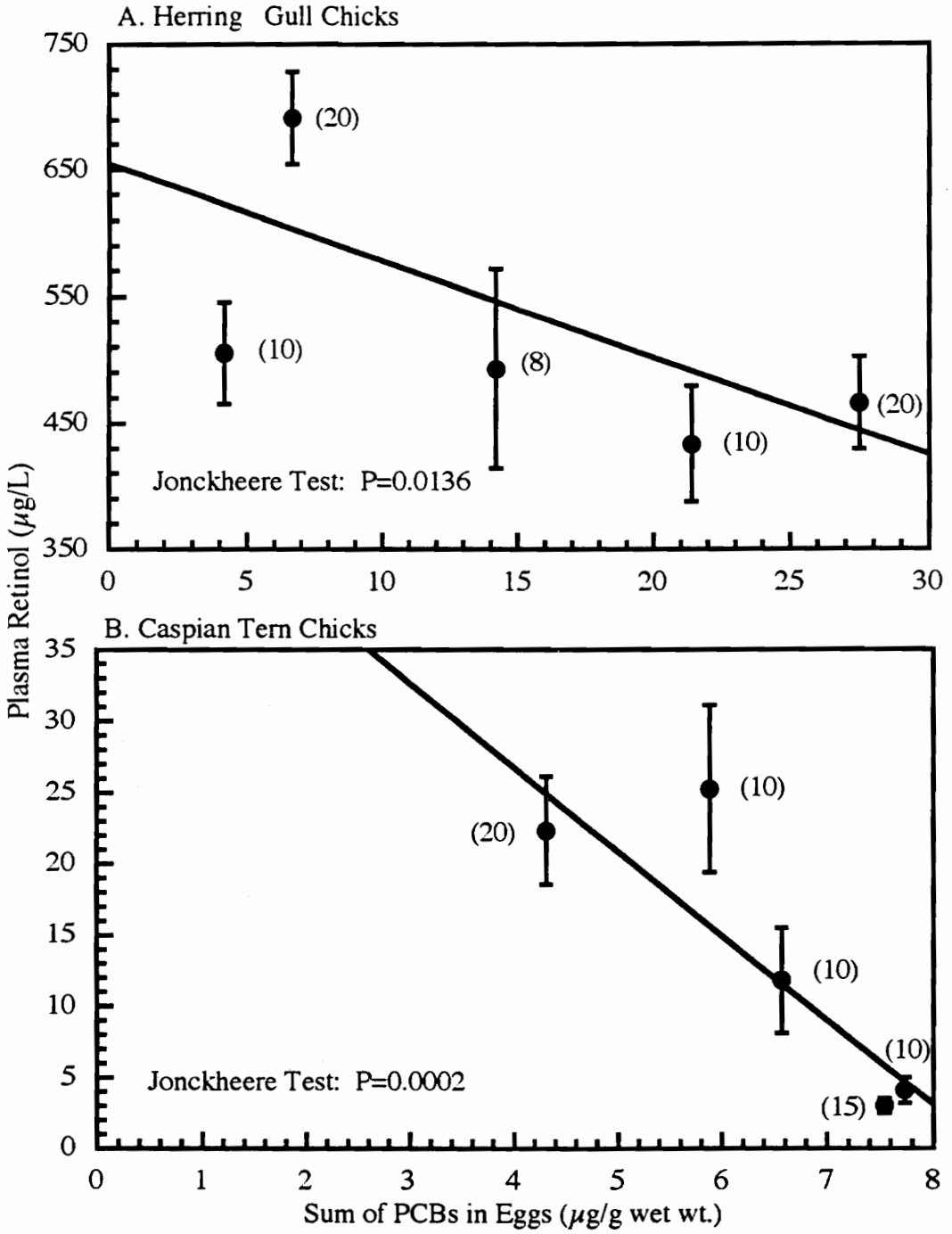

Fig. 4.5. Relationship between plasma retinol (vitamin A) and PCB contamination in herring gull (A) and Caspian tern (B) chicks from the Great Lakes and Lake Winnipeg during 1992-94. Closed circles indicate mean response for each site. Error bars indicate one standard error of the mean. Numbers in parentheses indicate sample sizes.

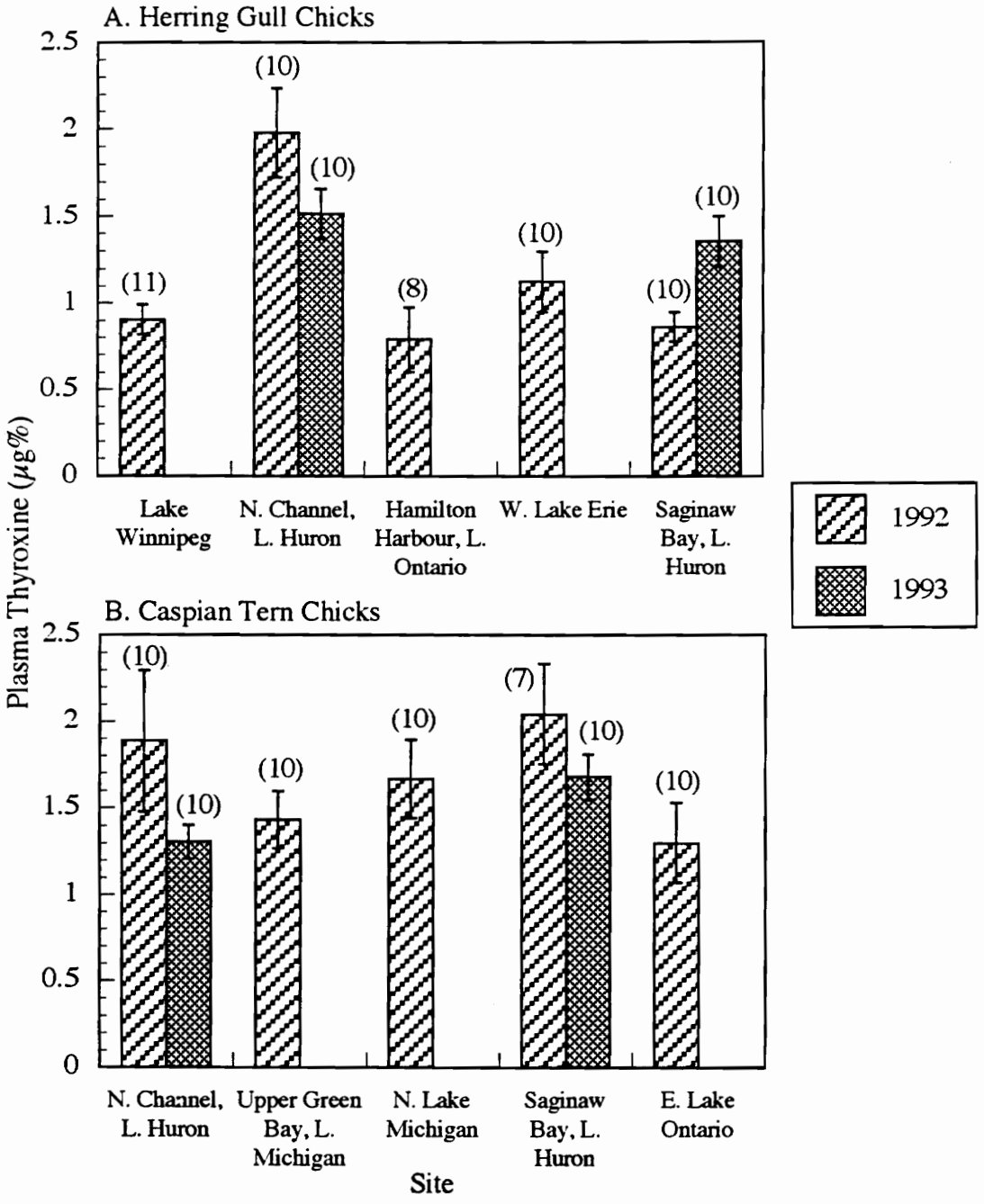

Fig. 4.6. Mean plasma thyroxine concentrations in herring gull (A) and Caspian tern (B) chicks from the Great Lakes and Lake Winnipeg during 1992-94. Error bars indicate one standard error of the mean. Numbers in parentheses indicate sample sizes.

Chapter 5. General Discussion: Ecoepidemiology and Evaluation of Biomarkers

Fish-eating colonial waterbirds of the Great Lakes historically have suffered the negative effects resulting from exposure to organochlorine contaminants. These effects include eggshell thinning caused by DDT and developmental effects caused by many contaminants, especially PCBs and TCDD (Peakall *et al.* 1980; Mineau *et al.* 1984; Fox and Weseloh 1987; Peakall and Fox 1987; Gilbertson 1988; 1989; Peakall 1988; Colborn *et al.* 1990; Fox *et al.* 1991; Gilbertson *et al.* 1991; Government of Canada 1991; Fox 1993). Reproductive failures occurred throughout the basin during the 1960's and early 1970's . Recent population-level effects associated with contaminants have been documented at highly contaminated sites (Ludwig 1979, Kubiak *et al.* 1989, Mora *et al.* 1993). While environmental contaminants have been associated with the disruption of a number of physiological systems in Great Lakes wildlife, before this study little or no effort had been devoted to determining impacts on the immune system. However, in laboratory animals, pollutants that are causing other health effects in Great Lakes wildlife also suppress immune function (Koller 1979, Caren 1981, Silkworth and Vecchi 1985, Thomas and Faith 1985, Luster *et al.* 1987, Vos and Luster 1989 Pruett 1994). The immune systems of developing and growing animals often are the most sensitive to the effects of PCBs and TCDD (Thomas and Faith 1985, Vos *et al.* 1989), and these effects on the developing immune system often persist long after exposure (Takagi *et al.* 1987, Holladay and Luster 1994).

Given the biologically significant effects that have been associated with HAHs in Great Lakes birds and the sensitivity of the immune system to these contaminants, this investigation studied whether immune function is suppressed in herring gulls and Caspian terns exposed to contaminants, especially HAHs, in the Great Lakes. This chapter evaluates the results of this study from the perspective of its two main objectives. The first objective was to use ecoepidemiological criteria to determine whether the immune systems of herring gulls and Caspian terns are affected by environmental contaminants in the Great Lakes. This chapter will evaluate effects on the structure and function of the T cell-mediated immunity, the structure and function of antibody-mediated immunity, and the absolute and relative numbers of white blood cells. The second objective was to evaluate the use of various immunological tests as biomarkers for contaminant-associated health effects in wild birds.

Ecoepidemiological Assessment of Immunotoxicity in Fish-Eating Birds of the Great Lakes

For ethical and financial reasons, manipulative field experiments in ecotoxicology often are not possible. However, releases of pollutants into the environment do cause negative impacts on living organisms, populations, and ecosystems. Pollution patterns are set by the locations of industrial facilities and the movement patterns of air and water. In the absence of manipulative experiments, ecoepidemiological criteria can be applied to establish what contaminants are responsible for biological effects and to allow for remediation of these problems (Fox 1991; Chapter 1). For epidemiologists, establishing causation does not require that a factor is a necessary and sufficient condition to produce an effect. Rather,

causal associations imply that a factor is part of a complex that increases the probability of an effect, and that reducing the factor reduces the probability of the effect. Ecoepidemiological criteria used to determine causal associations include

- **time order**-exposure precedes the effect;
- **strength of association**-the coincidence of the supposed cause and effect and the magnitude of the changes;
- **specificity**-a unique effect produced only by a single cause and a consistent effect that always accompanies a causal factor;
- **consistency upon replication**-the replication of the association between potential cause and effect when studied in different species, at different sites, during different years, by different investigators;
- **coherence**-agreement with current biological theory and the presence of a dose-response relationship;
- **predictive performance**-putative causal relationship leads to a deduction or prediction that is verified by further investigation;
- **probability**-the presence of a statistically significant relationship, although biological significance also must be evaluated.

All of these criteria do not need to be met to establish a causal association. In a particular situation, the criteria that support an association are weighed against the criteria that detract from it. Ecoepidemiological investigations can incorporate data from manipulative laboratory and field experiments as well as from field experiments where exposure regimes are less well-controlled by the investigators. This section evaluates three hypotheses about contaminant-associated immunosuppression using these ecoepidemiological criteria.

Suppression of T-Cell-Mediated Immunity

Within the constraints of this study, there was strong evidence for an association between persistent environmental contaminants and suppression of T-cell-mediated immunity in fish-eating birds of the Great Lakes. These effects were evident in both the structure (thymus mass) and the function (PHA skin test) of the T-cell-mediated branch of the immune system. The identity of the particular organochlorine(s) responsible for such suppression could not be determined because concentrations of different organochlorines were highly co-correlated. However, PCBs were the most likely cause for these effects because PCB concentrations were by far the highest of any immunosuppressive organochlorines. Strong support for this association came from the criteria of probability, strength of association, specificity, consistency, coherence, predictive performance, and time order (Table 5.1).

Using the criterion of probability, the Jonckheere test for ordered alternatives demonstrated that there was strong evidence that these associations were not caused by chance. There was very strong evidence that the PHA skin response decreased as several measures of organochlorine contamination increased ($P<0.001$; Table 4.2) and strong evidence that thymus mass decreased as liver EROD activity increased ($P=0.013$, Table 3.1). Beyond statistical significance, the strength of association criterion suggested that the magnitude of the changes in these variables was biologically significant. The PHA skin test was suppressed 30-45% at the most contaminated sites, and thymus mass was decreased 20-45% at the sites with highest EROD activity. In the case of the PHA skin test, there was strong spatial coincidence between organochlorine contamination and suppression of T lymphocyte function. The criterion of replication strongly supported these relationships. These effects were demonstrated in two species of fish-eating birds

(herring gulls and Caspian terns) and were replicated at highly contaminated sites and reference sites during multiple years (2-3).

The criterion of coherence also supported an association between contaminants and T-cell-mediated immunity. The Jonckheere test for ordered alternatives revealed monotonic (not necessarily linear) trends in the PHA skin test with respect to various organochlorines and in thymus mass with respect to liver EROD activity. Such associations are consistent with many laboratory experiments that have found severe impacts of HAHs on the structure and function of the T-cell-mediated branch of the immune system in birds and mammals (Vos and Van Driel-Grootenhuis 1972; Sharma *et al.* 1978; Hinsdill *et al.* 1980; Thomas and Hinsdill 1980; Clark *et al.* 1981, 1983; Silkworth and Grabstein 1982; Greenlee *et al.* 1985; Takagi *et al.* 1987; Nikolaidis *et al.* 1988a; Vos and Luster 1989; Fine *et al.* 1990; Lundberg *et al.* 1990; Andersson *et al.* 1991; Holladay *et al.* 1991; Neubert *et al.* 1991; Tomar *et al.* 1991; Blaylock *et al.* 1992). The field data from fish-eating birds of the Great Lakes also is consistent with the association between T lymphocyte function and organochlorines, especially HAHs, found in harbor seals and bottlenose dolphins (DeSwaart *et al.* 1994, Lahvis *et al.* 1995, Ross *et al.* 1995). Perinatal exposure to HAHs may also disrupt T-cell-mediated immunity in humans. In Inuit infants in the arctic region of Quebec, there was an association between increasing PCB and TCDD concentrations and reduced numbers of helper T lymphocytes (Dewailly *et al.* 1993). Infants with higher HAH exposure also had an increased incidence of middle ear infections during the first year of life. The effects of perinatal exposure to HAHs in humans may be long lasting. Children from Times Beach, Missouri, who were exposed to TCDD during development and (or) the first four years after birth had reduced numbers of helper T lymphocytes (Smoger *et al.* 1993).

The criterion of predictive performance also lent support to contaminant-association effects on T-cell-mediated immunity. The laboratory studies listed above were successfully used to predict the suppression of T-cell-mediated function and reduction in thymic mass in fish-eating birds at sites with high organochlorine contamination. The results of a pilot field study during 1991 were successfully used to predict suppression of the PHA skin test. This response was 60% lower in herring gull chicks from a highly contaminated site (Gull Island, Upper Green Bay) as compared to a reference site (Kent Island, Atlantic coast).

The specificity criterion refers to both a unique effect produced only by a single cause and a consistent effect that always accompanies a causal factor. One difficulty with examining the effects of environmental contaminants on the immune system is that many other factors such as nutrition, stress, infections, infestations, and genetics influence immune function. Hence, immunosuppression is not specific to pollutants. However, the fact that an association between contamination and T-cell-mediated immunity was so clearly demonstrated even in the presence of these confounding factors strengthens this association. Because suppression of T-cell-mediated immunity so consistently occurs after developmental exposure to HAHs in laboratory animals, the specificity criterion also supports this association in Great Lakes birds.

The time order criterion provided some support for this association because chicks were exposed to environmental contaminants throughout development and after hatch. This exposure occurred before T cell function and thymic mass were measured. Unfortunately, there are no data on immune function in Great Lakes fish-eating birds before the era of organochlorine pollution, so it cannot be determined whether these spatial patterns in immune function existed before this contamination.

Thus, this study provides strong evidence that environmental contaminants influence immune function in fish-eating birds of the Great Lakes. This association raises concerns because T-cell-mediated immunity plays both important regulatory and cytotoxic roles in combating infections. This importance is illustrated by the consequences of T-cell-mediated immunosuppression that occurs in humans with AIDS. Contaminant-associated immunosuppression on the level of individual birds could have significant population-level effects.

Suppression of Antibody-Mediated Immunity

Evaluation of this study using ecoepidemiological criteria did not support the hypothesis of contaminant-associated suppression of antibody-mediated immunity (Table 5.1). Neither structural (bursa mass and histology) or functional (sheep red blood cell antibody test) biomarkers showed consistent immunosuppression associated with contaminants, even though these variables exhibited significant differences among sites. Four criteria (probability, strength of association, coherence in the form of a dose-response relationship, and prediction from laboratory to field investigations) detracted from this hypothesis. Other criteria were indeterminate.

However, several factors may have confounded associations between contaminants and bursa mass and histology. Extensive bursal infestation with a fluke (*Cotylurus communis*) caused bursitis in herring gull chicks at all sites except for the reference site on the Atlantic coast, possibly obscuring effects of contaminants. In laboratory experiments with chicken embryos or young chicks, the size of the bursa and (or) the number of lymphoid cells in the bursa decreased in a dose-dependent fashion upon exposure to HAHs (Harris *et al.* 1976, Nikolaidis *et al.* 1988b, Andersson *et al.* 1991). In one study,

lymphoid depletion of the bursa was more sensitive to HAHs than that of the thymus (Nikolaidis *et al.* 1988b). Hence, the criteria of specificity and coherence suggest a possible association between contaminants and bursa mass. Examination of bursa mass and lymphoid cellularity in unhatched herring gull embryos might elucidate the effects of contaminants by eliminating confounding effects of infestation by flukes, differences in pre- and post-hatch contaminant exposure, and differences in post-hatch nutrition.

Several criteria detracted from the hypothesis of contaminant-associated suppression of B lymphocyte function. In both herring gull and Caspian tern chicks, antibody responses varied between sites and, in some cases, between years. This lack of association is consistent with laboratory studies showing that HAHs most consistently influence B cell function at doses that are higher than those required to suppress T cell function. Field investigations and studies using wildlife species in captivity also have shown less consistent associations between organochlorine contaminants and B lymphocyte functions. Harbor seals exposed to organochlorines showed reduced proliferation and antibody production by B lymphocytes (DeSwaart *et al.* 1994, Ross *et al.* 1995). However, organochlorines did not influence B lymphocyte proliferation in male bottlenose dolphins, although T cell proliferation was reduced (Lahvis *et al.* 1995).

Alteration of White Blood Cell Counts

Application of ecoepidemiological criteria to white blood cell counts in Caspian tern chicks and herring gull chicks and adults provided support for associations with contaminants, but the evidence was weaker than for T-cell-mediated immunity (Table 5.1). However, no criteria detracted from the hypothesis. The most consistent associations were found in herring gull adults. Counts of total and relative white blood cells can be viewed as

biomarkers that monitor the structure of the immune system. The probability, strength of association, coherence, and time order criteria clearly supported associations between contaminants and white blood cell variables. With respect to strength of association, there was good coincidence between the distribution of contaminants and effects on white blood cells. The magnitudes of changes in white blood cell counts were biologically significant. With respect to coherence, the Jonckheere test for ordered alternatives revealed dose-response relationships between contaminants and white blood cells. The time order criterion supported this association because birds were exposed to environmental contaminants before white blood cell variables were measured

The criteria of replication, specificity, and prediction provided indeterminate evidence. In herring gulls, many of the effects on white blood cell counts in adults were not seen in chicks. In herring gull chicks that were later necropsied, there was an association between HG-TEQs in the liver and the heterophil/lymphocyte ratio. However, in the herring gull chicks used for the functional tests, there was no association between HG-TEQs in eggs and this ratio. However, Caspian tern chicks did show an association between organochlorine contamination in eggs and the heterophil/lymphocyte ratio. Hence, effects on the heterophil/lymphocyte ratio were replicated in some instances but not in others. The specificity criterion was indeterminate because so many factors other than contaminants can influence white blood cell counts. However, in laboratory experiments TCDD and PCBs cause changes in the numbers of circulating lymphocytes at relatively high doses that produce other toxic effects (Vos and Van-Driel Grootenhuis 1972, Vos and Luster 1989). Some associations between HAH-exposure and white blood cell variables were apparent in fish-eating birds of the Great Lakes. The prediction criterion was difficult to apply because contaminants might be expected to increase or decrease white blood cell

counts, depending on the degree of immunosuppression and the dynamic nature of white cell responses to infection. Some immunosuppressed animals may suffer from recurrent infections, leading to increased white blood cell counts as the body attempts to fight these infections. In more severely immunosuppressed animals, white blood cell counts may be reduced before infection and unable to proliferate after infection.

Evaluation of Structural and Functional Biomarkers

Biomarkers are biochemical, physiological, or histological changes that measure effects of or exposure to toxic chemicals. Immunological biomarkers are important for assessing health effects in wild animals. This study compared two different types of biomarkers: those that measure biological structures (organ masses and histology, and blood cell variables) and those that measure biological function (PHA skin test and sheep red blood cell antibody test). Since function usually depends on structure, relationships between these two types of biomarkers were explored. The PHA skin test showed associations with organochlorine contaminants most clearly. This test provided a simple, effective, nonlethal measure of T cell function. Thymus mass and white blood cell counts also showed strong associations. Bursa mass and antibody production were not clearly associated with organochlorines. Hence, measurement of both structural and functional biomarkers was important for investigating relationships to contaminants--the case for such associations would have been weaker if only structural or only functional biomarkers had been assessed. Indeed, the National Toxicology Program includes both structural and functional variables in a tiered system for testing chemicals for immunotoxic potential (Luster *et al.* 1988b, 1994). None of the structural or biochemical biomarkers provided

surrogate measures for direct tests of T-cell-mediated or antibody-mediated immune function.

Although some biomarkers did not reveal associations with organochlorine contaminants in this study, these variables still are useful for assessing the health of wild birds. All the biomarkers used in this study have excellent potential for assessing the health of birds in other studies where exposure levels, contaminants, or species are different from the present study. Almost all of the variables showed statistically and biologically significant differences among different colonies, suggesting that they were sensitive to factors such as environmental contaminants, nutrition, stress, weather.

Table 5.1. Ecoepidemiological evaluation of immunotoxic effects of environmental contaminants on fish-eating birds of the Great Lakes.[1]

Criterion	Hypothesis		
	Suppression of T-Cell-Mediated Immunity	Suppression of Antibody-Mediated Immunity	Alteration of White Blood Cell Counts
Probability	+	-	+
Strength of Association	+	-	+
Specificity			
In Effects of a Cause	+	?	+
In Causes of an Effect	?	?	?
Consistency upon Replication	+	?	+
Coherence			
Theoretical Plausibility	+	?	+
Factual Compatibility	+	?	+
Biological Coherence	+	?	+
Dose-Response	+	-	+
Predictive Performance			
Laboratory to Field	+	-	?
Field to Field	+	I.D.	I.D.
Time Order	+	?	+

[1] Supports: +
Detracts: -
Indeterminate: ?
Insufficient Data: I.D.

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Vita

Keith Alan Grasman was born to Gerald and Helen Grasman of Silver Spring, Maryland, on January 17, 1967. He graduated as class valedictorian of Paint Branch High School in Burtonsville, Maryland, in June 1985. He pursued a B.S. in biology at Calvin College in Grand Rapids, Michigan, and graduated with honors in May 1989.

After marrying Suzanne Henderson of Gary, Indiana, on 12 August 1989, he began graduate work in wildlife toxicology in the Department of Fisheries and Wildlife Sciences at Virginia Polytechnic Institute and State University. In March 1992, he completed a M.S. degree for a project entitled "Effects of Lead Ingestion on Immune Function in Quail." He then pursued a Ph.D. degree at VPI &SU, investigating the effects of environmental contaminants on fish-eating birds of the Great Lakes. His work was supported by a National Science Foundation Pre-Doctoral Fellowship and a Society of Environmental Toxicology and Chemistry/ Procter and Gamble PreDoctoral Fellowship. He also received a predoctoral fellowship from the International Association for Great Lakes Research/ Mott Foundation. He also was awarded a fellowship from the International Association for Great Lakes Research and the Mott Foundation. Before completing his Ph.D., Keith returned to Calvin College to teach biology and environmental studies for two years. He continued his research on Great Lakes toxicology and immunotoxicology. Just before completing his Ph.D., Keith accepted a position as Assistant Professor of Environmental Health Effects at Wright State University in Dayton, Ohio.

Keith is a member of the Society of Environmental Toxicology and Chemistry, the Ecological Society of America, The Wildlife Society, the Great Lakes Wildlife Bioeffects Group, the International Association for Great Lakes Research, The Colonial Waterbird