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CHEMICAL AND BIOLOGICAL POLLUTION CONTRIBUTE TO THE IMMUNOLOGICAL PROFILES OF FREE-RANGING HARBOR SEALS

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Abstract—Polychlorinated biphenyls and other persistent organic pollutants have been associated with immunotoxicity and outbreaks of (infectious) disease in marine mammals by rendering them vulnerable to infection by pathogens such as viruses and bacteria. In an immunotoxicological study of free-ranging harbor seals (*Phoca vitulina*), we obtained samples of blood and blubber from seal pups that were live-captured from two remote and two near-urban sites in British Columbia, Canada, and Washington state, USA. Using these samples, we quantified hematology, innate immune function, adaptive immune function, and polychlorinated biphenyl accumulation. While controlling for confounding factors (age, sex, and condition), univariate correlations between phagocytosis ($r^2 = 0.30$, $p = 0.002$), respiratory burst ($r^2 = 0.45$, $p = 0.000$), T-lymphocyte function ($r^2 = 0.16$, $p = 0.028$), lymphocyte signaling ($r^2 = 0.17$, $p = 0.025$), and lymphocyte counts ($r^2 = 0.29$, $p = 0.002$), and polychlorinated biphenyl concentrations suggested chemical-associated immunotoxicity. Principal component analysis of immunological endpoints provided additional evidence of immunotoxic effects in seals. However, principal component analysis also identified a noncontaminant-related factor by distinguishing between seals inhabiting urban versus remote sites, with results being consistent with increased pathogen exposure. Elevated fecal coliform concentrations in water, and observations of terrestrial spill-over pathogens in local seals, further support the notion of biological pollution at these sites. Although our study highlights the role that environmental contaminants might play in rendering marine mammal populations vulnerable to disease through immunotoxicity, it also suggests that biological pollution represents an emerging conservation concern.

Keywords—Harbor seals *Phoca vitulina* Polychlorinated biphenyls Immunotoxicity Biological pollution

INTRODUCTION

During recent decades, disease-related mass mortalities have affected several marine mammal populations around the world, leading to significant conservation concerns. Morbilliviruses, in particular, have been notable for their role in the deaths of thousands of harbor seals (*Phoca vitulina*) in northwestern Europe in 1988 and 2002; thousands of Baikal (*Pusa sibirica*) and Caspian (*Pusa caspica*) seals in 1987 to 1988 and 2000, respectively; and several thousand striped dolphins (*Stenella coeruleoalba*) in the Mediterranean Sea in 1990 to 1992 [1–3]. Periodic reports of outbreaks of leptospirosis among California sea lions (*Zalophus californianus*) [4], further underscore the extent to which pathogens play a role in morbidity and mortality of free-ranging marine mammals.

Evidence suggests that environmental contamination by polychlorinated biphenyls (PCBs) and structurally related compounds (e.g., polychlorinated dibenzo-*p*-dioxins and furans) might have contributed to some of these events through toxicity at the level of the immune system [5]. Many marine mammal species are at particular risk of immunotoxicity because of their high trophic level, long life span, and limited metabolic capacity to eliminate PCBs, resulting in the bioaccumulation of elevated levels of these compounds. In addition, reproductive transfer of the lipophilic PCBs, through fat-rich milk produced from the mother's blubber [6], exposes young marine mammals at a sensitive developmental stage for the immune system.

The immunotoxic effects of PCBs have been mechanistically documented in a number of laboratory studies and are most pronounced when exposure takes place early in life (prenatal, postnatal, or both periods) [7]. Effects on the adaptive immune system include reduced thymus and spleen weights; changes in T-lymphocyte subsets, number, and functionality; decreased antibody responses of B lymphocytes; and suppression of immunization-induced proliferation of lymphocytes [8–11]. Polychlorinated biphenyls can affect innate immunity by decreasing phagocytosis by leukocytes and macrophages and decreasing the activity of natural killer cells [12–14]. Reduced immune responses as a result of PCB-associated immunotoxicity have been associated with an increase in susceptibility to disease in laboratory animals [15], in wildlife species including birds and fish [16,17], and in humans [18].

Despite the logistical and ethical challenges associated with immunotoxicological studies in marine mammals, a weight of evidence supports the idea of chemical contaminant–associated immunotoxicity in pinnipeds and cetaceans. In two groups of captive harbor seals, fed either North Sea herring or more (PCB-)contaminated Baltic Sea herring, decreased mitogen-

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and antigen-induced proliferation of lymphocytes, decreased antibody responses, and lower natural killer cell activity in the Baltic group [19] were consistent with observations in PCB-exposed laboratory rodents. In addition, elevated levels of PCBs in harbor seals, harbor porpoises (*Phocoena phocoena*), and striped dolphins that died during virus-associated mass mortalities in northwestern Europe and the Mediterranean Sea, respectively, compared with healthy individuals suggest increased susceptibility to disease because of immunotoxicity [20,21].

In a preliminary study of a group of free-ranging seals in British Columbia, Canada, a relationship was observed between PCB concentrations and the adaptive immune response of harbor seals [22]. The objective of this study was to assess immune status in a larger group of free-ranging harbor seals, from a broader geographical region, covering a wider range of contaminant concentrations, and with an expanded immunological testing suite.

MATERIALS AND METHODS

Sampling

Pacific harbor seals (*P. vitulina richardsi*), aged 3 to 5 weeks, were captured at four sites in British Columbia (BC), Canada, and Washington state (WA), USA, by two different techniques. First, individual seal pups in a rocky intertidal area of Hornby Island (BC; 49°30′N, 124°35′W) were captured by hand with a selective and rapid approach by small craft [23]. Seals hauled out on the mud flats of Sea Island (BC; 49°12′N, 123°9′W), Smith Island (WA; 48°19′N, 122°49′W), and Gertrude Island (WA; 47°13′N, 122°35′W) were captured in groups with a large beach seine net [24]. Seals were manually restrained while collecting blood from the extradural vein, with a 3.2-cm 18-gauge needle, into ethylenediaminetetraacetic acid- and heparin-containing tubes (for hematology and for isolation of peripheral blood mononuclear cells and leukocytes, respectively; both Becton-Dickinson, Franklin Lakes, NJ, USA). Samples were kept at 4°C until processing (within 24 h). Blubber (for contaminant analysis) was sampled by 8-mm biopsy punches (Acuderm, Fort Lauderdale, FL, USA). In addition, body weight, sex, length, girth, and nursing status were recorded to estimate age, condition, and basic overall health. The time spanning capture and sampling was documented to avoid handling-related stress influences. After sampling, pups were released at the capture site. All procedures were carried out under the auspices of the respective animal care committees and scientific research permits for researchers in British Columbia (Fisheries and Oceans Canada Animal Care Committee according to guidelines from the Canadian Council on Animal Care; Fisheries and Oceans Canada Scientific Research Permit; and University of Victoria Animal Care Committee protocol 036-03) and Washington state (U.S. Marine Mammal Protection Act Permit 835).

Mitogen- and thymosin α_1-induced cell proliferation

Peripheral blood mononuclear cells were assessed for their proliferative responses according to methods adapted from De Swart et al. [25]. Heparinized whole blood was diluted one-on-one with RPMI-1640 containing 10 international units (IU)/ml of heparin (both from Gibco, Burlington, ON, Canada). Blood was subsequently gently layered onto Lymphoprep™ (Biolynx, Brockville, ON, Canada) to isolate the peripheral blood mononuclear cells by density gradient centrifugation (30 min at 600 *g* at room temperature). Peripheral blood mononuclear cells were washed three times and counted, and the concentration was adjusted into RMPI-1640 Glutamax supplemented with 10% fetal bovine serum (Cansera, Etobicoke, ON, Canada), penicillin–streptomycin (100 IU and 100 μg/ml, respectively; Gibco), and 2×10^{-5} M 2-mercaptoethanol (Sigma-Aldrich, Oakville, ON, Canada). Cells were cultured in Falcon™ flat-bottom 96-well plates (BD Biosciences, Mississauga, ON, Canada) at 10^5 cells per well in a constant environment of 37°C, 5% CO_2 in air, with or without stimulant. Two mitogens, concavalin A (ConA) and lipopolysaccharide (LPS), were used at concentrations found to be optimal by previous studies [26] of 1 and 5 μg/ml, respectively (both from Sigma-Aldrich). Thymosin α_1 (bovine, Sigma-Aldrich), a highly conserved thymic hormone, was used at an optimal concentration of 6.25 ng/ml (on the basis of average circulatory values reported for other species and preliminary experiments). The thymidine analogue 5-bromo-2′-deoxyuridine was added after 48 h. After a total 68-h incubation, cell proliferation was determined by colorimetric 5-bromo-2′-deoxyuridine incorporation enzyme-linked immunosorbent assay (Roche Diagnostics, Laval, QC, Canada). The relative response of stimulated cells versus nonstimulated cells is reported as a stimulation index (SI).

Phagocytosis and respiratory burst

Phagocytosis and respiratory burst were analyzed as described in De Guise et al. [27]. Briefly, heparinized whole blood was centrifuged for 10 min at 250 *g*, and the cell pellet was collected. Red blood cell lysis, by shock with NH_4Cl, provided a leukocyte sample. Leukocytes were resuspended in Hanks' Balanced Salt Solution (Gibco), washed three times, counted, and adjusted to a concentration of 2×10^6. For phagocytosis, leukocytes were incubated with fluorescent latex beads (Molecular Probes, Eugene, OR, USA) at 100 beads per leukocyte. Free beads were removed by layering the cell suspension on a 3% albumin gradient and centrifuging for 8 min at 150 *g* and 4°C. The fluorescence of approximately 10,000 leukocytes was quantified by flow cytometry and reported as the percentage of cells that had taken up at least one bead. Respiratory burst was quantified by incubating leukocytes (for 15 min at 37°C in the dark) with 5 μM 2,7-dichlorofluorescin diacetate (Molecular Probes), a probe that becomes fluorescent on production of H_2O_2. Subsequently, the oxygen-dependent pathway of phagocytes was activated with 10^{-9} phorbol myristate acetate (Sigma-Aldrich). Labeled cells were kept in the dark until flow cytometric analysis of approximately 10,000 leukocytes and quantified by the increase in activity in phorbol myristate acetate–stimulated cells compared with unstimulated cells, reported as an SI.

Contaminant analysis

Blubber samples of approximately 100 mg were analyzed for 34 (mono- and di-*ortho*) PCBs with IUPAC numbers 17, 18, 28, 31, 33, 44, 49, 52, 70, 74, 87, 95, 99, 101, 105, 110, 118, 128, 132, 138, 149, 151, 153, 156, 170, 180, 187, 191, 194, 195, 201, 205, 206, and 209 by Trent University (Peterborough, ON, Canada), as described elsewhere [28]. Briefly, samples were mixed with anhydrous sodium sulfate, extracted from a glass column with 50:50 dichloromethane:hexane, and quantified by gas chromatography with an electron capture detector. Procedural blanks and a National Institute for Standards and Testing (NIST 1588) cod liver oil reference material

were analyzed for quality control/quality assurance purposes. Detection limit substitutions were made for PCB congeners that were not detected for cases in which more than 70% of the seals had detectable values, whereas congeners in which 70% or less of the seals were detected were replaced by zeros. The percent lipid was determined with the gravimetric lipid determination by weight of extraction method to calculate lipid-normalized concentrations of contaminants.

Hematology and fecal coliform analyses

The Central Lab for Veterinarians (Langley, BC, Canada) carried out hematology tests on blood smears from ethylenediaminetetraacetic acid–containing blood samples. White blood cell counts were reported as total white blood cell (WBC) count, and the numbers and proportions of the different circulating WBC subpopulations (neutrophils, monocytes, lymphocytes, eosinophils, and basophils). Fecal coliform concentrations in water were provided by Environment Canada (Hornby Island), the Greater Vancouver Regional District (Sea Island), and the Washington State Department of Health (Smith and Gertrude Islands) and were reported as the number of fecal coliform (FC) bacteria per 100 ml. These values represented the fecal coliform monitoring area closest to our seal sampling sites and were taken during the period of seal sampling (or, in the case of Hornby Island, as available).

Data analysis

Data were presented as the mean plus or minus the standard error of the mean. Further statistical analysis was carried out with SPSS® 12.0 for Windows (SPSS, Chicago, IL, USA). Comparisons of immune responses between sexes were done by Student's *t* test (two-tailed), assuming equal variances. Linear regression analyses (r^2) were applied to associations between immune status, confounding factors, and contaminant concentrations. For lymphocyte function, the SI was multiplied by 10 and log transformed for regression against contaminant concentrations. A cumulative measure of adaptive immune response (total SI) for each animal was calculated with the sum of mean-adjusted SI of cell proliferation in response to ConA, LPS, and thymosin α_1. Associations that failed the criterion for normality (Lilliefors significance; i.e., lymphocyte count) were assessed by Spearman's rho correlation. Results were considered significant at $p \leq 0.05$.

Because the basis of measurement differed for the immune parameters, each parameter was divided by the maximum value observed for the parameter before principal component analysis (PCA). Hematological values were scaled to the maximum WBC count because individual counts were fractions of the total WBC count, rather than independent measures. Arcsine transformation (calculation of the square root followed by the arcsine) of this proportional data set returned it to a normal distribution and was then autoscaled before PCA. Missing data (WBC values for one Smith seal and adaptive responses for one Hornby seal) were replaced by the average value of seals from the same location. Varimax rotation was used to position the axes to maximum possible sum of the variances of the loadings, while maintaining orthogonal eigenvectors. Sample scores for significant principal components (PCs) and the geometric mean regression of PC1 and PC2 (as the exploratory value of a combination of PC1 and PC2) [29] were correlated to contaminant concentrations and confounding factors to assess which were contributing to differences in immune function among seals.

RESULTS AND DISCUSSION

A number of virus-associated mass mortalities have taken place among marine mammal populations inhabiting industrial coastal areas during recent decades, suggesting that chemical contamination could have played a role in the occurrence and severity of these events. The relatively high concentrations of PCBs in marine mammals and their established immunotoxicity in a variety of species [18,30,31] highlight the risk that this chemical class, in particular, might pose to the health of marine mammals in many parts of the world [32].

Immunotoxicity associated with chemical exposure can affect both the quality and the quantity of circulating WBCs, thereby diminishing defenses against invading pathogens. In this study, we characterized immune function in a total of 31 harbor seal pups, which were similar in body weight (20.1 ± 0.5 kg) and therefore estimated age [23], with an equivalent sample size between sexes (females $n = 17$, males $n = 14$). This limited the influence of potentially confounding factors. In addition, we did not detect a correlation between any of the measures of immune function and body weight ($p > 0.05$; results not shown), or contaminant concentrations and body weight ($r^2 = 0.011$, $p = 0.578$). No significant differences in immune function and contaminant concentrations between sexes (Student's *t* test, $p > 0.05$; results not shown) were found; therefore, sexes were combined for further analysis. Contaminant concentrations varied widely among seals (396–6,587 μg/kg lipid wt; $n = 31$) on the basis of the sum of 34 PCB congeners, with the most contaminated animals originating from Gertrude Island in the southern Puget Sound (Fig. 1). Our study design therefore provided a relatively controlled setting within which confounding factors were eliminated or minimized while a range of contaminant concentrations was maximized.

Measures of the adaptive immune response of seals correlated negatively with PCB concentrations. The proliferative responses of T lymphocytes, but not of B lymphocytes (mimicked by stimulation with the polyclonal activators ConA and LPS, respectively; Fig. 2A and B), were significantly decreased in more contaminated animals. This is consistent with an immunotoxic mechanism of action attributed to the aryl hydrocarbon receptor (AhR) in laboratory animals [33] and in vitro–exposed marine mammal cells [34]. Although the sensitivity of T lymphocytes to immunotoxic compounds is well established, few studies have evaluated lymphocytes recovered from free-ranging marine mammals. In a small group of cetaceans ($n = 5$), decreased T-lymphocyte proliferation was attributed to either PCBs or DDT [35]. Levin et al. [22], on the other hand, reported recently that PCBs might also increase the proliferative response of (suboptimally stimulated) lymphocytes in harbor seals ($n = 18$). Therefore, immunotoxic compounds might both suppress and stimulate, but stimulation could reflect a response of only the most immunocompetent cells or the effects of hormesis at low contaminant concentrations [36,37].

Lymphocyte signaling, an endpoint not previously assessed in free-ranging marine mammals, was also significantly reduced in more contaminated seal pups (Fig. 2C), as evidenced by the decrease of in vitro responsiveness to thymosin α_1. Thymosin α_1 induces interleukin-2 (IL-2) secretion, an important lymphocyte growth factor, and stimulates synthesis of the IL-2 (high-affinity) receptor, both of which are important in lymphocyte proliferation after pathogen recognition. Response to this immunomodulatory molecule can therefore

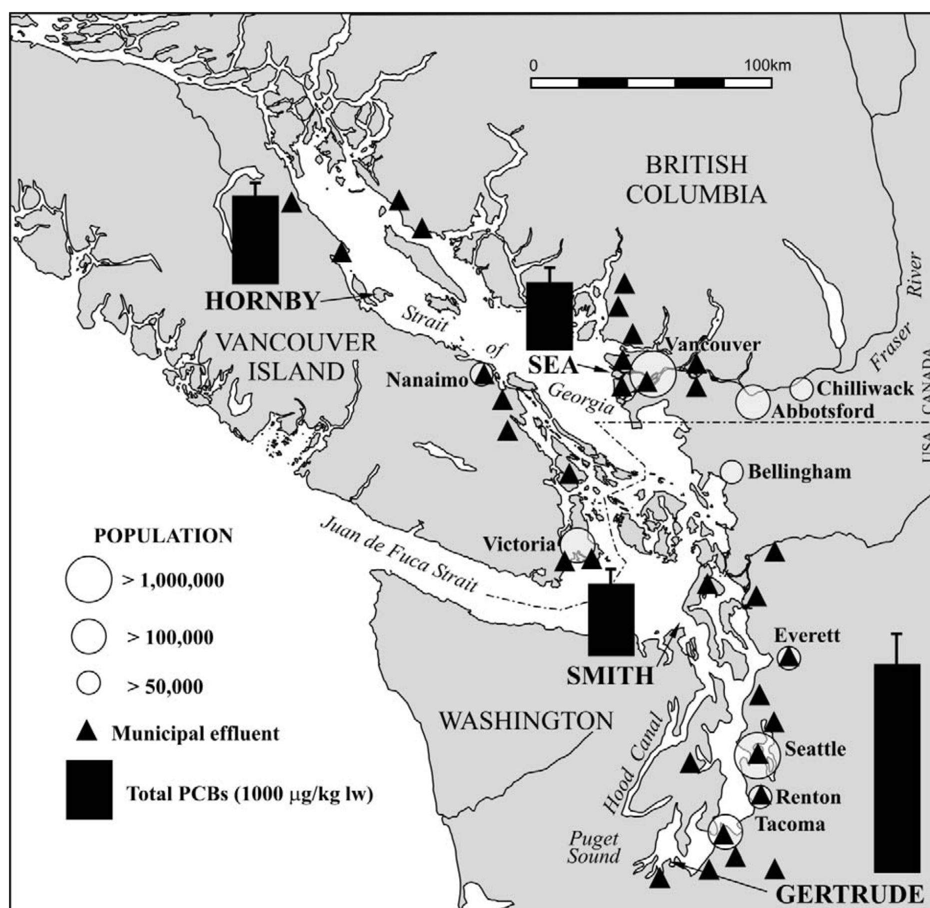

Fig. 1. Harbor seal pups sampled at four island locations in the transboundary region between British Columbia, Canada, and Washington state, USA, had a range of blubber polychlorinated biphenyl (PCB) concentrations (396–6,587 μg/kg lipid wt). The sites spanned remote (Hornby and Smith Islands; $n = 8$ and 8, respectively) and near-urban (Sea and Gertrude Islands; $n = 7$ and 8, respectively) environments, with the latter proximate to urban centers, municipal effluent, and agricultural activities.

serve as an indirect measure of lymphocyte signaling. Although PCBs could be mediating a disruption of IL-2 signaling via the thymosin α_1 receptor, our results more likely reflect an effect at the level of IL-2. Polychlorinated biphenyls are known to disrupt cytokines [38,39] and cytokine regulation [40] through AhR-dependent mechanisms of action, and harbor seal peripheral blood mononuclear cells exposed in vitro to PCBs were shown to decrease in functionality at least in part because of a disruption of cell signaling and cytokine (IL-1 and IL-2) production [41].

Reduced overall lymphocyte function, as captured by a total stimulation index (Fig. 2D), indicates that seals exposed to contaminants had lower overall (mean-adjusted) lymphocyte functionality. In addition to this reduced functionality, the more contaminated seals had decreased circulating concentrations (Spearman's rho $r = -0.534$; $p = 0.002$) and decreased percentages of lymphocytes (Pearson $r = -0.26$; $p = 0.004$) in the WBC counts. Because PCBs appear to be affecting both the quality and quantity of lymphocytes, the adaptive immune system of the more contaminated seals, as a whole, might be less able to respond to infectious agents.

Reduced phagocytic activity of neutrophils and monocytes in more contaminated animals (Fig. 2E) suggested another immunotoxic effect of contaminants. However, increased respiratory burst (Fig. 2F), an intracellular process in which reactive oxygen intermediates are produced to kill and digest ingested pathogens, by the same cell types, could indicate an immunotoxic enhancement. These observations are supported by observed decreases in phagocytosis in marine mammal cells [42,43] and increased respiratory burst in human cells in response to in vitro exposure to PCBs [44]. Altered function of phagocytic cells appear to be largely explained by AhR-independent mechanisms of action and might result from oxidative stress, disturbance of particle uptake, or interference with antioxidant enzymes [45,46]. A PCB-associated disruption of innate immunity is of concern because these cell types (primarily neutrophils but also monocytes) represent a rapid, nonmemory-based immunological defense against many types of invading pathogens, such as bacteria.

Given the complexity of the interacting components of the immune system, a PCA model was used to characterize overall immune status in our harbor seals. The PCA model separated seals into three overlapping clusters, with the remote Hornby and Smith Island (H/S) seals projecting in the upper right quadrant, the near-urban Sea Island (V) seals forming an intermediate group to the left of axis center, and the near-urban Gertrude Island (G) seals located in the lower left quadrant (Fig. 3). The seal samples with missing values projected near the center of the cluster for each location and, accordingly, the substitution of average values had not affected the separation by location. Principal component 3 was not used for interpretation because of a low eigenvalue and the strong influence of a few outlier samples (not shown).

The corresponding variable projections indicated a pattern

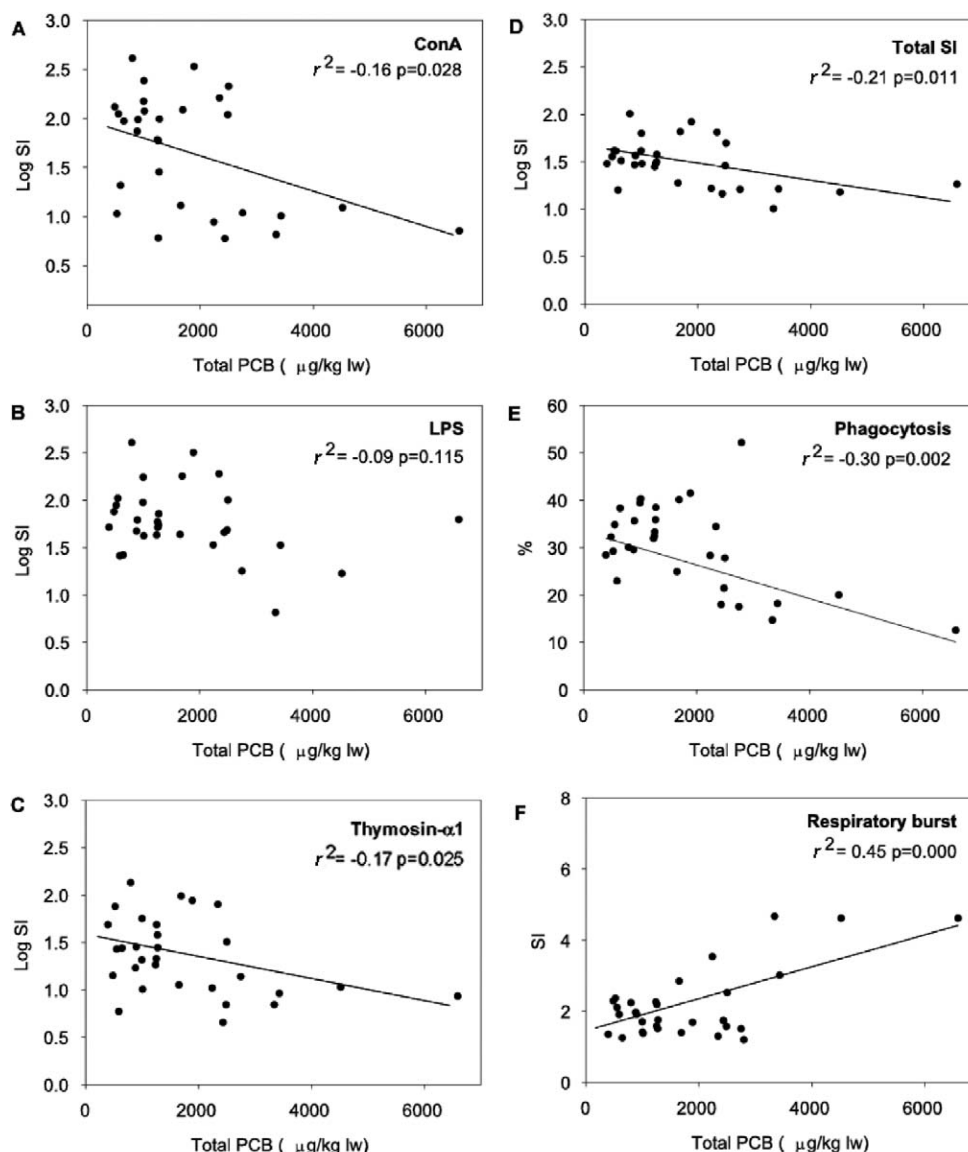

Fig. 2. Lymphocyte function in harbor seals was negatively associated with polychlorinated biphenyl (PCB) concentrations (μg/kg lipid wt) in response to a T-lymphocyte mitogen concavalin A (ConA) and the IL-2-stimulating hormone thymosin α_1, but not in response to the B-cell mitogen lipopolysaccharide (LPS; **A**–**C**). The total stimulation index (SI), representing the mean-adjusted cumulative lymphocyte response (ConA, LPS, and thymosin α_1) of each seal, indicates that the more contaminated seals had reduced lymphocyte performance (**D**). Increasing PCB concentrations were also significantly associated with reduced particle uptake (**E**) and elevated respiratory burst (**F**) by phagocytic cells. Lipid weight (lw).

of low lymphocyte and phagocyte functionality and high respiratory burst activity for the more contaminated G, compared with less contaminated H/S, animals, which is consistent with the univariate associations found in this study. The low importance of lymphocyte/monocyte numbers (projecting in the upper left and close to axis center, respectively, indicating little influence on the model) and relatively higher neutrophil counts in G seals suggest that decreased functionality rather than decreased cell numbers characterize the changes in immune status of contaminated animals. The scores of the loading variables in PC1 and PC2 and the geometric mean regression of PC1 with PC2 correlated significantly with PCB concentrations (Table 1), but this relationship was strongest with PC2. The contribution of immunotoxic contaminants to the differences in immune function among seals therefore appears to be reflected by PC2.

On the other hand, PC1 provided a clear separation between seals sampled at near-urban locations (G/V) versus the more remote (H/S) sites, independent of PCB concentrations. Within the near-urban sampling locations, PCB concentrations are much higher in seals from G than V, suggesting that their (horizontal) clustering is not associated with chemical pollution. The two sites share features, however; both are influenced by freshwater runoff and are in close proximity to major human population centers (greater Seattle, WA, USA, and Vancouver, BC, Canada, respectively), agriculture, and sewage treatment plants (for the latter, plants are within 5–10 km, compared with >30 km for remote H/S sites; Fig. 1). The variable projections of high neutrophil and eosinophil counts that could not be explained by univariate associations with PCBs (both Spearman's rho, $r = 0.222$; $p = 0.237$ and $r = 0.281$; $p = 0.132$, respectively) but that characterize PC1 suggest that seals sampled at near-urban sites experience a higher exposure to bacteria and parasites, respectively. In addition, increased respi-

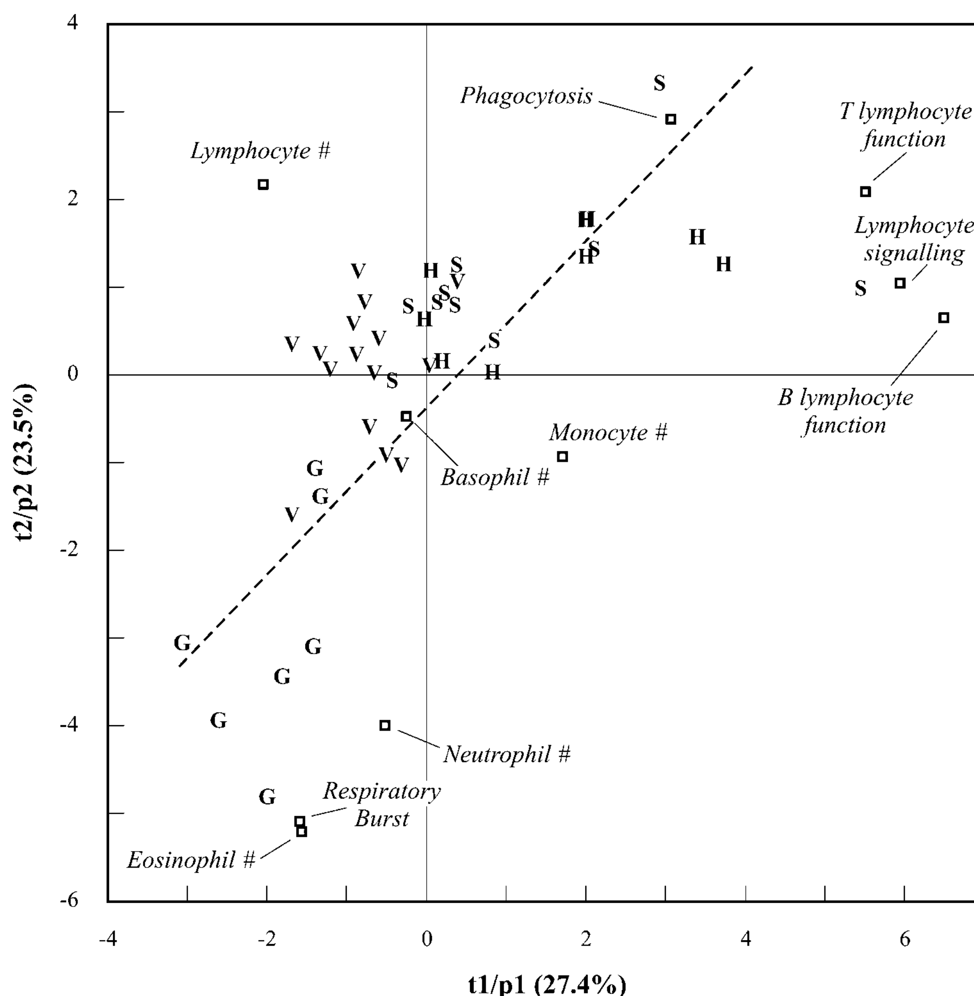

Fig. 3. Varimax rotated projections of a principal component analysis (PCA) model on the basis of 10 immune function variables revealed that two key factors influenced the immunological profiles of harbor seals. PC2 separated harbor seal pups into three overlapping groups: Gertrude Island (G), Washington, USA; Sea Island (V), British Columbia, Canada; and Hornby (Vancouver, Canada) and Smith Islands (H/S), Washington, USA. G seals were characterized by relatively high respiratory burst and low phagocyte and lymphocyte function, whereas H/S seals were the opposite. The significant relationship between PC2 and polychlorinated biphenyl (PCB) concentrations (Table 1) confirmed univariate results that indicated that seals from more contaminated sites in this region suffer from immunotoxicity. The horizontal separation of urban (G and V) and nonurban (H/S) seals in PC1, coupled with the variable projections, suggests that proximity to sources of biological pollution also contributes to the immunological profiles of seals (Table 2). The dashed line represents the geometric mean regression relationship between the samples in PC1 and PC2.

Table 1. Regression analysis of principal component (PC) 1 and 2, the sum of polychlorinated biphenyls (Σ PCBs), body weight, and the time between capture and sampling of the seals indicates that PC1 and PC2 are correlated to contaminant concentrations and not to potentially confounding factors

	PC1 (eigenvalue 2.7)	PC2 (eigenvalue 2.4)	Geometric mean regression of PC1 with PC2
Σ PCBs	$r^2 = -0.15$ $p = 0.032$	$r^2 = -0.50$ $p = 0.000$	$r^2 = 0.15$
Body weight	$r^2 = 0.01$ $p = 0.572$	$r^2 = 0.01$ $p = 0.604$	
Sampling time	$r^2 = 0.02$ $p = 0.451$	$r^2 = 0.05$ $p = 0.169$	

ratory burst could indicate an up-regulation of mechanisms involved in bacterial destruction. This immunological profile, combined with the separation between near-urban and remote locations, strongly suggests that biological pollution could be the underlying factor.

To characterize biological pollution in the vicinity of our four sampling locations by means of organic waste input, we obtained FC counts from the responsible public health agencies. Fecal coliform bacteria, living in the digestive tract of warm-blooded animals and excreted in their feces, are routinely used as an indicator of biological (fecal) pollution of drinking and recreational waters. The FC counts were high and fluctuating at both of the near-urban (G/V) seal sampling sites, whereas they were low to undetectable at both remote (H/S) sites (Table 2), which is consistent with a role of biological pollution in explaining the differences in immunological profiles between groups of harbor seals. The source of FC at the seal sampling locations is not clear, but could be from either human sources (sewage discharge, septic fields, storm water drainage), agricultural activities (runoff from livestock

Table 2. Fecal coliform (FC) measurements in water samples from near the seal sampling sites in British Columbia, Canada, and Washington State (WA), USA, indicate a higher level of biological pollution at the near-urban sites of Gertrude Island (G), WA, and Sea Island (V), BC, compared with the remote Hornby (H), BC, and Smith Islands (S), WA. Similar seal abundance at the four locations suggest that FC was likely not of seal origin, but rather a result of nearby human activities. FC data from Environment Canada (H), Washington State Department of Health (S, G), and Greater Vancouver Regional District (V)

Location	Haul-out size (No. of seals)[a]	Population density (people/km^2)[b]	Agricultural activities (yes/no)[b]	Mean FC/100ml (range)[c]
Hornby Island (H)	≥1,500	25	No	3.3 (<2–5)
Smith Island (S)	~500	None	No	5.6 (<2–13)
Gertrude Island (G)	≥500	97	Yes	15.6 (<2–110)
Sea Island (V)	≥1,000	715	Yes	32.5 (23–55)

[a] Data for H from Olesiuk [48], S/G/V from Jeffries et al. [49].
[b] Data for H/V from Statistics Canada (year 2001, www.statcan.ca). Population density for V includes the cities of Vancouver and Richmond. Data for G from the coastal Puget Sound area, USA (year 2000) [50].
[c] Data from Environment Canada (H), Washington State Department of Health (S/G), and Greater Vancouver Regional District (V). H: mean value of nine sampling stations sampled once each during February 2004 (low tide; no data available for the summer of 2003–2004). S: mean value of three sampling stations at Point Partridge, sampled once each during September 2003 (low tide). G: mean value of nine sampling stations in Wycoff Shoals, sampled once each during September 2003 (low tide). V: 30-d mean of five samplings at Iona Beach, September 2003; range from 30-d mean FC values from May to September 2003 (tide unknown).

and soil fertilization), or wildlife. However, all sites have a similar abundance of seals (Table 2), and wildlife generally contributes only chronically low levels of FC (H. Osachoff, Environment Canada–Pacific and Yukon Laboratory for Environmental Testing, North Vancouver, BC, Canada, personal communication). Therefore, the source of FC at the G and V sites is likely related to the upstream contributions of humans, pets, and livestock into these two coastal areas.

Evidence for a role of biological pollution also arises from the recent isolation of a number of bacterial pathogens from rehabilitated seals from British Columbia and Washington state (1999–2003) that are typically associated with sewage and agricultural runoff. These include *Escherichia coli*, *Enterococcus* spp., *Salmonella* spp., *Clostridium difficile*, *Clostridium perfringens*, *Klebsiella* spp., *Coxiella burnetti*, *Listeria monocytogenes*, and *Pseudomonas* sp. (S. Raverty, Animal Health Centre, Abbotsford, BC, Canada, unpublished observations). Furthermore, the presence of the protozoa *Toxoplasma gondii* (associated with cat feces in freshwater runoff), *Giardia* (from canids and other terrestrial wildlife), and *Sarcocystis neurona* (diagnosed in horses in British Columbia, but also affecting cats and other terrestrial vertebrates, i.e., opossums) in harbor seals within our study area provides direct evidence of spillover of terrestrial pathogens into coastal marine mammals ([47]; J. Gaydos, University of California-School of Veterinary Medicine, Davis, CA, USA, personal communication; S. Raverty, Animal Health Centre, Abbotsford, BC, unpublished observations).

Our study documents a negative relationship between immune function and PCB concentrations in free-ranging harbor seals in British Columbia, Canada, and Washington state, USA. These observations are further supported by a weight of evidence from mechanistic studies with laboratory animals and captive seals. In addition, although our study was not explicitly designed to assess the effect of biological pollution, we detected a distinct immunological signature that was consistent with elevated pathogen exposure in seals inhabiting near-urban, coliform-contaminated areas. With an impaired immune response, seals from more contaminated coastal regions (e.g., Puget Sound) could be particularly vulnerable to infection by new pathogens introduced into their habitat by anthropogenic activities.

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