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Immune Function and Organochlorine Pollutants in Arctic Breeding Glaucous Gulls

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Abstract. Organochlorine contaminants (OCs) are known to affect the immune systems of wildlife, and in this study we assessed the relationship between blood concentration of different OCs and measurements relevant to immune status and function in arctic breeding glaucous gulls (*Larus hyperboreus*). In 1997 and 2001, we counted white blood cells (heterophils and lymphocytes) from blood smears, and in 2000 and 2001 we injected two novel nonpathogenic antigens (diphtheria and tetanus toxoids) into the pectoral muscle of gulls and measured the primary antibody responses. We then related these measurements to the blood concentrations of three pesticides (hexachlorobenzene [HCB], oxychlordane, and *p,p′*-dichlorodiphenyldichloroethylene) and seven different polychlorinated biphenyl congeners (PCB 101, 99, 118, 153, 138, 180, and 170). There were significant or near significant positive relationships ($0.1 > p > 0.001$) between most persistent OCs and the levels of heterophils in the blood for both sexes in 1997 and for male gulls in 2001. Similarly, levels of all persistent OCs and lymphocytes were positively related ($0.1 > p > 0.001$) in both sexes in 1997. This suggests that OCs are causing alterations to immune systems, which may decrease their efficiency and make the birds more susceptible to parasites and diseases. In female gulls, the antibody response to the diphtheria toxoid was significant and negative for HCB ($p < 0.01$) and weaker, but significant, for oxychlordane ($p < 0.05$), suggesting that OCs were causing an impairment of the humoral immunity. Various OCs have been linked to negative effects in our study population, including decreased survival and reproduction, and this study suggests that such compounds also affect immune status and function.

Infectious organisms are overwhelmingly abundant, and both species and individual numbers highly exceed those of free living organisms (Smith and Roberts 1981). It is consequently of no surprise that they are considered particularly important in population regulation and the evolution of free-living animals (Grenfell and Dobson 1995; Hamilton 2001). The immune system of hosts is of major importance in decreasing the impact of infectious organisms, but various environmental contaminants are known to be immunotoxic to wildlife, thus potentially increasing susceptibility to infectious diseases (Vos and Luster 1989; Grasman *et al.* 1996, 2002). However, few studies of free-living adult birds have examined the relationship between contaminant burdens and immune function. We studied the relationship between blood concentrations of various organochlorine contaminants (OCs) and variables relevant for immunologic status and function in glaucous gulls (*Larus hyperboreus*). The glaucous gull is an arctic top predator that has repeatedly been found to have high levels of atmospherically transported organochlorine pollutants such as polychlorinated biphenyls (PCBs) and *p′p′*-dichlorodiphenyldichloroethylene (DDE) (Bourne and Bogan 1972; Gabrielsen *et al.* 1995; Cleeman *et al.* 2000; Braune *et al.* 2002). Moreover, at Bear Island in the European Arctic, aberrant behaviours and unexplained deaths have been reported among glaucous gulls with high OC concentrations since the early 1970s (Bourne and Bogan 1972; Gabrielsen *et al.* 1995). More recently, increased blood concentrations of OCs have been linked to decreased feeding efficiency, decreased reproduction and survival, high parasite loads, and fluctuating asymmetry in wing feathers in this population (Sagerup *et al.* 2000; Bustnes *et al.* 2001a, 2002, 2003). How OCs affects glaucous gulls are poorly understood. The levels of PCBs and DDE are not sufficiently high to be lethal by themselves (Gabrielsen *et al.* 1995), and Henriksen *et al.* (2000) suggested that glaucous gulls were not particularly sensitive to Ah-receptor mediated effects of OCs. However, Sagerup *et al.* (2000) found a positive relationship between the intensity of nematode parasites and liver concentrations of various OCs, especially heavy chlorinated PCB congeners, and suggested that this resulted from the immunotoxic properties of the OCs followed by increased susceptibility to parasitic infection. A potential pathway through which OCs exert their effects in glaucous gulls is thus through impairment of the immune system.

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Because avian species are known to respond to infectious organisms by increasing the numbers of white blood cells (Davies 1981; Hawkey *et al.* 1983; Averbeck 1992), we counted white blood cells (WBCs), both heterophils and lymphocytes, from blood smears. Heterophils are part of the innate immune defense system, play an important role during the initial stages of most infections, and are the primary means of controlling bacterial infections (Roitt *et al.* 1998). In contrast, lymphocytes are the main cell types in the adaptive immune response (Janeway *et al.* 1999). White blood cell counts are general, nonspecific indicators of health status, and high levels may indicate either increased infection or high immunocompetence (e.g., Siva-Jothy 1995; Sheldon and Verhulst 1996; Norris and Evans 2000). A better way to examine the ability of an animal to mount a specific immune reaction against new infections is by measuring the strength of its response against a nonpathogenic immune challenge. The objectives of this study were as follows: (1) to examine the relationships between OC concentrations in the blood and immune status as reflected by counts of heterophils and lymphocytes and (2) to examine the relationships between OC concentrations in the blood and the ability to mount immune reactions as reflected by the strength of the response against novel antigens (diphtheria and tetanus toxoids).

Materials and Methods

The study was carried out on Bear Island (74° 30′ N, 19° 01′ E) in 1997, 2000, and 2001. Details about the study area have been described in Bustnes *et al.* (2000, 2001a). In late May and early June, breeding glaucous gulls were caught on their nests between 5 and 10 days into incubation using a nest trap (Bustnes *et al.* 2001a), and blood was sampled from the wing vein (about 10 mL) with a syringe. We measured bill length, bill height (±0.1 mm), skull length (head plus bill), tarsus (±0.5 mm), and wing length (±1 mm), and performed a principal component analysis on these measurements for the purpose of obtaining a composite measurement of body size (i.e., the first principal component of PC1). However, skull length was the measurement most strongly related to PC1 ($0.77 < r < 0.88$ for both sexes in all years), and for simplicity we used only skull length as a body size measurement in the analyses (see also Coulson and Thomas 1983). The birds were weighted to the nearest 10 g. As an index of body condition, we used body weight, controlling for body size (skull length) in the statistic models (García-Berthou 2001). Sex was determined by size; male gulls were larger than female gulls (see Bustnes *et al.* 2001a for details).

White Blood Cell Counts

One blood smear was prepared from each blood sample. The smear was immediately fixed in methanol and stored for later analysis. Blood smears were stained by the May-Grünewald-Giemsa staining method. Between 1997 and 2001, the procedure for counting blood cells in smears was changed in the laboratory. In 1997, red blood cells (RBCs) and WBCs) were counted in two independent areas scanned at × 400 magnification, whereas in 2001, counts were made in three independent areas at × 1000 magnification. The WBCs were classified as lymphocytes or heterophils based on their appearance (Hawkey and Dennett 1989). We calculated the lymphocyte-to-RBC and the heterophil-to-RBC ratios by averaging the ratios from the two or three counts from each individual. Because of the differences between methods used in 1997 and 2001, the WBC counts were not quantitatively compared between years, and the years were analyzed separately. To test the reliability of our WBC-to-RBC ratios, we correlated the ratios obtained from the two or three counting areas on each slide (see Hanssen *et al.* 2003). In 1997, the WBC-to-RBC ratios were strongly correlated between the counting areas on the slides ($r_s = 0.734$, $p < 0.0001$), whereas in 2001, the correlations were lower between the three areas counted ($0.401 < r_s < 0.480$) but still highly significant ($p < 0.0001$). This suggests a reasonably high repeatability for our WBC-to-RBC ratios.

Immune Challenge

To quantify the individual's ability to respond immunologically to foreign antigens, we injected 0.15 mL diphtheria–tetanus vaccine (SBL Vaccin AB, Stockholm, Sweden), diphtheria toxoid 38 Lf (flocculation entities), and tetanus toxoid 7.5 Lf mixed with the adjuvant aluminium phosphate (5mg/mL) into the pectoral muscle of birds trapped between 5 and 10 days into incubation. At this time, prevaccination blood samples were collected to examine background antibody response to the enzyme-linked immunosorbent assay (ELISA). The birds were captured again 14 to 17 days later and a new blood sample (1 mL) was collected. This was done in 2000 and 2001, the timing of which is close to the peak titres for primary immunoglobulin (Ig)G responses against nonpathogenic antigens in birds (Hasselquist *et al.* 1999; Hanssen *et al.* 2004).

ELISA Assay

We measured humoral immune system activation as the antigen-specific antibody levels in the gull sera using a standard ELISA previously developed for red-winged blackbirds (for details of the methods, see Hasselquist *et al.* 1999). The assay has been proven to work for other passerines and waders (Råberg *et al.* 2000; Hasselquist *et al.* 2001; D. Hasselquist unpublished data, 2001–2003), and in the study we showed that it works well also for gulls. The ELISA method provides sensitive measures of the amount of antibodies that specifically bind to the antigen.

We used a diluent of 1% powdered milk in 0.01 mol/L phosphate-buffered saline (PBS) to produce 1:1800 dilutions for the tetanus plates and 1:900 dilutions for the diphtheria analyses of the preimmunization and postimmunization serum samples. To avoid between-plate variation, we ran all analyses on 96-well ELISA plates for each of the two antigens and analyzed all plates on the same day for each of the 2 years samples. Antigens (diphtheria and tetanus toxoid) were diluted to 3 μg/mL in carbonate buffer (0.15 mol/L, pH 9.6) and added to plates in 100 μ well. After incubation overnight at 4°C and blocking with 3% milk powder in PBS, 100 μL serum sample (see above) were added to each well and incubated overnight at 4°C. Plates were then washed in PBS and Tween 20; as a result, only glaucous gull antibodies that had specifically bound to the antigen (diphtheria or tetanus) fixed to the sides of the wells remained on the ELISA plate. Next, 100 μL of the secondary antigen, rabbit-anti–red-winged blackbird (RaRW) diluted 1:1500 in PBS, were added to each well. The RaRW had previously been produced by immunizing rabbits with purified red-winged blackbird IgM and IgG (Hasselquist *et al.* 1999, 2001). After a second incubation and wash, a peroxidase-labeled goat-anti-rabbit antibody (Kirkegard & Perry, Gaithersburg, Maryland), diluted 1:2000 in PBS, was added to each well. After a second incubation (1 hour at 37°C) and wash, peroxidase substrate (2,2-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid, ABTS, catalogue no. A1888; Sigma), and peroxide were added, and the plates were immediately transferred to a V_{max} (Molecular Dynamics, Sunnyvale, CA) kinetics ELISA reader. The plates were read at 30-second intervals for 10 minutes using a 405-nm

wavelength filter. All antibody concentrations are given as the standardized slope of the substrate conversion (in $10^{-3} \times$ optical densities [OD]/minute; mOD $\times$ min^{-1}), with a higher slope indicating a higher concentration of antigen-specific antibodies in a sample. Preinjection serum samples from each individual were run in duplicate to investigate each individual's background level of antigen-specific antibodies. For each individual, postinjection serum samples were added to the plate in duplicate, and the average of these was our measure of antibody titre for each dilution. We ran at least 2 wells with blank samples on each plate. (These wells were treated in the same way as the test sample wells except that no bird serum was added.) As our measure of preimmunization and postimmunization antibody titres of individual birds, we subtracted the mean value of these blanks from the measured antibody concentration. We ran 11 standard samples covering the range of antibody titres for the injected birds on each plate. The differences between the standard curves were used to adjust preinjection and postinjection antibody titres to control for between-plate variations. For each individual, we then subtracted the preinjection titre from the postinjection titre to obtain values of the primary antibody response, which we used as a measure of the humoral immune responsiveness in further analyses

Analyses of Organochlorines

The analyses were carried out in the Environmental Toxicology Laboratory at the Norwegian School of Veterinary Science/National Veterinary Institute. Samples of whole blood (about 8 g) were weighed, and an internal standard (CB-29 and CB-112) was added. The methods used for extraction (cyclohexane and acetone), clean-up (with sulphuric acid), and quantification (gas chromatographic) of the samples were first described by Brevik *et al.* (1978), and modifications have been described by Andersen *et al.* (2001). Percent extractable fat was determined gravimetrically. Aliquots of the final extracts were injected on an Agilent gas chromatograph–electron capture detector (GC-ECD) (6890 series; Agilent, Wilmington, DE). The GC was equipped with two capillary columns (SPB-5 and SPB-1701; Supelco Bellefonte, PA). Quantification was done within the linear range of the detector. Detection limits were defined as three times the background noise. The following PCB-congeners were determined: IUPAC nos. 101, 99, 118, 138, 153, 170, and 180. Other compounds analyzed included hexachlorobenzene, oxychlordane, and DDE. The detection limits for the individual PCBs ranged from 0.01 to 0.02 ng/g wet weight (w/w) for HCB, from 0.005 to 0.01 for oxychlordane, and from 0.01 to 0.02 ng/g for DDE. GC conditions, temperature program, and quality-assurance procedures have been described by Andersen *et al.* (2001). Analytic standards of the laboratory were certified by participation in international intercalibration tests. Certified international reference materials (CRM 349 and 350, ICES cod liver oil and mackerel oil) were analyzed regularly and results were within the given ranges. The laboratory was accredited for these analyses according to the requirements of NS-EN 45001 (Norwegian and European standard) and ISO/IEC Guide 25.

Because OCs mainly partition to the lipid portion of the plasma, and we measured OC in whole blood, the measurement may have been influenced by the packed-cell volume (hematocrite value). We therefore examined the variation in packed-cell volume between individuals in 2001 by centrifuging a capillary tube with blood from each individual on a Compur Mini Centrifuge (Bayer Diagnostic, GmbH, Ames Microspin, Typ: 6500 200, München, Germany) at 11,500 rpm for 4 minutes 15 seconds. The variation in packed cell volume was low ($\bar{x}$ = 42.5 $\pm$ 0.5% standard error, Table 1).

We used the concentration of OCs in whole blood (w/w) as a measure of exposure to OCs. Other studies have suggested a stability of the blood levels of OCs in incubating glaucous gulls under stable conditions such as found at Bear Island, indicating that blood concentration is a reliable, relative (compared with other individuals) measurement for body burden of a given individual (Henriksen *et al.* 1998; Bustnes *et al.* 2001b).

Statistics

The different persistent OCs were highly correlated (Table 2), and it is therefore difficult to extract the effects of individual compounds from a mixture. However, some variation existed in the correlations between compounds, and our justification for conducting separate analyses for all OCs was that it may indicate which compounds in a mixture of OCs that were important as agents causing negative effects, especially if they could repeatedly be linked to effect parameters (Bustnes *et al.* 2003). Hence, some compounds may show stronger effects than others on immune-related parameters. Because concentrations of the different OCs were obtained from the same blood samples, one might normally adjust alpha values for multiple comparisons. After Rothman (1990), however, we did not conduct such adjustments when testing the predictions about the relationship between OC levels and immune status and function. A central argument for not adjusting the p values is that doing so would increase the likelihood of accepting false null hypotheses and thus disqualify further examination of potentially important relationships between specific compounds and immunologic effects. We do, however, report p values from two-tailed tests because we did not have unidirectional predictions.

Statistical analyses were carried out using SAS software (SAS Institute, Cary, NC). For comparisons between WBC indices and OC levels, values were $\log_{10}$ transformed to approximate a normal distribution. However, the data on the antibody response to vaccine injection could not be normalized using logarithmic transformations, and consequently we rank transformed the antibody response and then used general linear models on the ranks (Conover and Iman 1981; Potvin and Roff 1993). Standard error is given for all means. Two outliers were removed from the data based on evaluation of studentized residuals (Schlotzhauer and Littell 1991).

Immune status and function may be influenced by various factors including nutritional and reproductive status (e.g., Sheldon and Verhulst 1996). In this study, we tested if a number of such potential confounding variables influenced any of the immunologic variables. Because the ELISA analyses of antibody response took place in 2 different years, we included *year* (variable no. 1) in models for all OCs to control for possible between-assay differences. For the WBC data, in contrast, the 2 years (1997 and 2001) were analyzed separately (see above). Other variables included *breeding location* (variable no. 2) because previous studies in this glaucous gull population have demonstrated differences in OC levels and diets between different types of breeding areas, i.e., between seabird cliffs and those from other areas (Bustnes *et al.* 2000). Moreover, we also tested if *body condition* (body mass controlled for body size and sex, variable no. 3) *incubation stage* (number of days since egg laying, variable no. 4), and *egg laying date* (variable no. 5) influenced any immunologic variables. We analyzed immune status and function in relation to 11 different OCs (including sum-PCB), and to decrease the amount of statistical tests, we tested if variables no. 2 through 5 showed any relationship to the immunologic variables. If there were no positive or negative relationships, we did not control for these variables in the statistical models that estimated the relationships between immunologic variables and OCs.

Results

Comparisons of OC Levels Between Years and Sexes

In 1997, male gulls had much higher concentrations of persistent OCs compared to female gulls, whereas in 2001 the

Table 1. Hematocrit values (packed blood cell volumes) of glaucous gulls from Bear Island in 2001

N	Mean	SD	Median	CV	Range	Quartile 1	Quartile 3
101	0.425	0.054	0.433	12.68	0.350	0.399	0.461

CV = coefficient of variance.

Table 2. Correlation coefficients for relationships between different OCs in the blood of glaucous gulls at Bear Island in 1997 and 2001

OC	HCB	Oxychlordane	DDE	PCB-101	PCB-99	PCB-118	PCB-153	PCB-138	PCB-180	PCB-170
1997										
HCB										
Oxychlordane	0.848									
DDE	0.798	0.817								
PCB-101	0.518	0.326	0.394							
PCB-99	0.881	0.924	0.806	0.405						
PCB-118	0.896	0.920	0.844	0.394	0.968					
PCB-153	0.846	0.926	0.792	0.335	0.968	0.967				
PCB-138	0.892	0.917	0.805	0.407	0.982	0.980	0.975			
PCB-180	0.827	0.926	0.778	0.365	0.937	0.945	0.972	0.960		
PCB-170	0.817	0.891	0.764	0.397	0.905	0.924	0.934	0.930	0.948	
Sum-PCB	0.869	0.935	0.809	0.376	0.976	0.980	0.994	0.990	0.983	0.948
2001										
HCB										
Oxychlordane	0.793									
DDE	0.891	0.834								
PCB-101	0.644	0.734	0.691							
PCB-99	0.862	0.909	0.875	0.810						
PCB-118	0.897	0.910	0.922	0.797	0.978					
PCB-153	0.813	0.907	0.868	0.807	0.980	0.966				
PCB-138	0.861	0.897	0.887	0.808	0.991	0.979	0.987			
PCB-180	0.743	0.875	0.821	0.802	0.944	0.927	0.983	0.956		
PCB-170	0.777	0.900	0.839	0.821	0.963	0.991	0.991	0.972	0.993	
Sum PCBs	0.825	0.907	0.875	0.814	0.984	0.973	0.999	0.992	0.983	0.992

HCB = Hexachlorobenzene.
OC = Organochlorine contaminant.
PCB = Polychlorinated biphenyl.

difference between sexes was lower (Table 3). Among female birds, the concentrations of most OCs were comparable in 1997 and 2001, apart from a significant increase in the level of HCB in 2001 (Table 3). In male birds in 2001, the OC levels were similar between years except for significant decreases, especially in oxychlordane concentrations but also in concentrations of PCB-99 and PCB-118 (Table 3).

Immune Status and Function in Relation to Potential Confounding Variables

Body condition was not related to heterophile indices in 1997 ($p = 0.50$) and 2001 ($p = 0.93$) or to lymphocyte indices in 1997 ($p = 0.20$) and 2001 ($p = 0.94$). Body condition also did not influence responses to tetanus ($p = 0.33$) or diphtheria toxoid ($p = 0.63$). There were no differences in any of the immunologic variables between the breeding areas ($0.26 < p < 0.93$), and incubation stage (number of days since egg laying) had no effects on any of the immunologic variables ($0.40 < p < 0.94$). Moreover, egg-laying date did not have any effect on any immunologic variables ($0.30 < p < 0.99$). All of these variables were therefore excluded from further analyses.

White Blood Cell Indices

In 1997, the heterophil indices in female gulls were significant and positively related to the blood concentration of all OCs (Table 4 and Fig. 1) except PCB-101 and PCB-170 (one outlier was removed because it had a very low heterophile index). For male birds, similar significant relationships were found for oxychlordane and most PCB congeners (Table 4 and Fig. 2).

Lymphocyte indices in 1997 were positively related to concentrations of several OCs. In female birds, all OCs except PCB-101 and PCB-99 were significantly related to lymphocyte indices. HCB, oxychlordane, and DDE showed strong relationships to lymphocyte indices (Table 4 and Fig. 1). In male birds, especially oxychlordane, but also most PCB congeners, showed significant relationships to lymphocyte indices (Table 4 and Fig. 2).

In 2001, there were no significant relationships between

Table 3. Comparison of mean concentrations (w/w) of OCs in the blood of glaucous gulls in 1997 and 2001

	1997 (N = 110)		2001 (N = 101)		
Source	ng g^{-1}	SE	ng g^{-1}	SE	*p* value[a]
Female gulls					
HCB	11.27	1.30	17.90	1.42	<0.0001
Oxychlordane	16.70	2.46	11.81	1.16	0.78
DDE	61.50	8.64	72.67	6.31	0.057
PCB-101	1.04	0.09	1.14	0.17	0.71
PCB-99	16.74	1.93	16.22	1.74	0.83
PCB-118	32.0	5.28	27.24	3.01	0.63
PCB-153	106.61	22.28	114.37	13.85	0.16
PCB-138	62.67	10.87	65.80	8.02	0.46
PCB-180	53.87	8.73	57.51	8.74	0.33
PCB-170	16.74	2.56	16.67	2.25	0.64
Male gulls					
HCB	23.64	2.60	22.27	1.74	0.56
Oxychlordane	31.94	3.73	14.99	1.24	<0.0001
DDE	113.28	10.27	122.29	12.21	0.56
PCB-101	1.30	0.11	1.53	0.17	0.23
PCB-99	34.54	4.34	21.91	2.18	0.015
PCB-118	65.23	7.72	41.06	4.27	0.008
PCB-153	237.74	33.80	177.34	21.55	0.45
PCB-138	134.82	19.14	97.84	10.85	0.24
PCB-180	100.51	11.13	90.27	10.64	0.78
PCB-170	31.85	3.25	25.32	3.02	0.14

[a]Results of Student *t* tests on $\log_{10}$-transformed data.
HCB = Hexachlorobenzene.
OC = Organochlorine contaminant.
PCB = Polychlorinated biphenyl.
w/w = Wet weight.

Table 4. The relationships between heterophil and lymphocyte indices ($\log_{10}$ transformed) and blood concentration of different OCs ($\log_{10}$ transformed) in glaucous gulls in 1997

	Female Gulls (N = 35)			Male Gulls (N = 30)		
Source	*p* value	Trend	R^2	*p* value	Trend	R^2
Heterophile indices						
HCB	0.0060	+	0.207	0.115		
Oxychlordane	0.0087	+	0.191	0.0206	+	0.177
DDE	0.0008	+	0.291	0.22		
PCB-101	0.56			0.95		
PCB-99	0.0121	+	0.176	0.05	+	0.127
PCB-118	0.0126	+	0.174	0.065	+	0.116
PCB-153	0.0207	+	0.152	0.0226	+	0.172
PCB-138	0.0139	+	0.170	0.0776	+	0.107
PCB-180	0.0207	+	0.152	0.0291	+	0.159
PCB-170	0.0536	+	0.108	0.0439	+	0.137
Sum-PCB	0.0167	+	0.161	0.0342	+	0.150
Lymphocyte indices						
HCB	0.0082	+	0.188	0.0776	+	0.106
Oxychlordane	0.0095	+	0.112	0.0044	+	0.256
DDE	0.0094	+	0.182	0.0352	+	0.149
PCB-101	0.54			0.31		
PCB-99	0.0731	+	0.091	0.028	+	0.161
PCB-118	0.0456	+	0.113	0.041	+	0.141
PCB-153	0.0455	+	0.113	0.008	+	0.226
PCB-138	0.0484	+	0.110	0.0843	+	0.103
PCB-180	0.0294	+	0.132	0.0229	+	0.172
PCB-170	0.0507	+	0.108	0.0460	+	0.135
Sum PCBs	0.0403	+	0.118	0.0205	+	0.177

HCB = Hexachlorobenzene.
OC = Organochlorine contaminant.
PCB = Polychlorinated biphenyl.

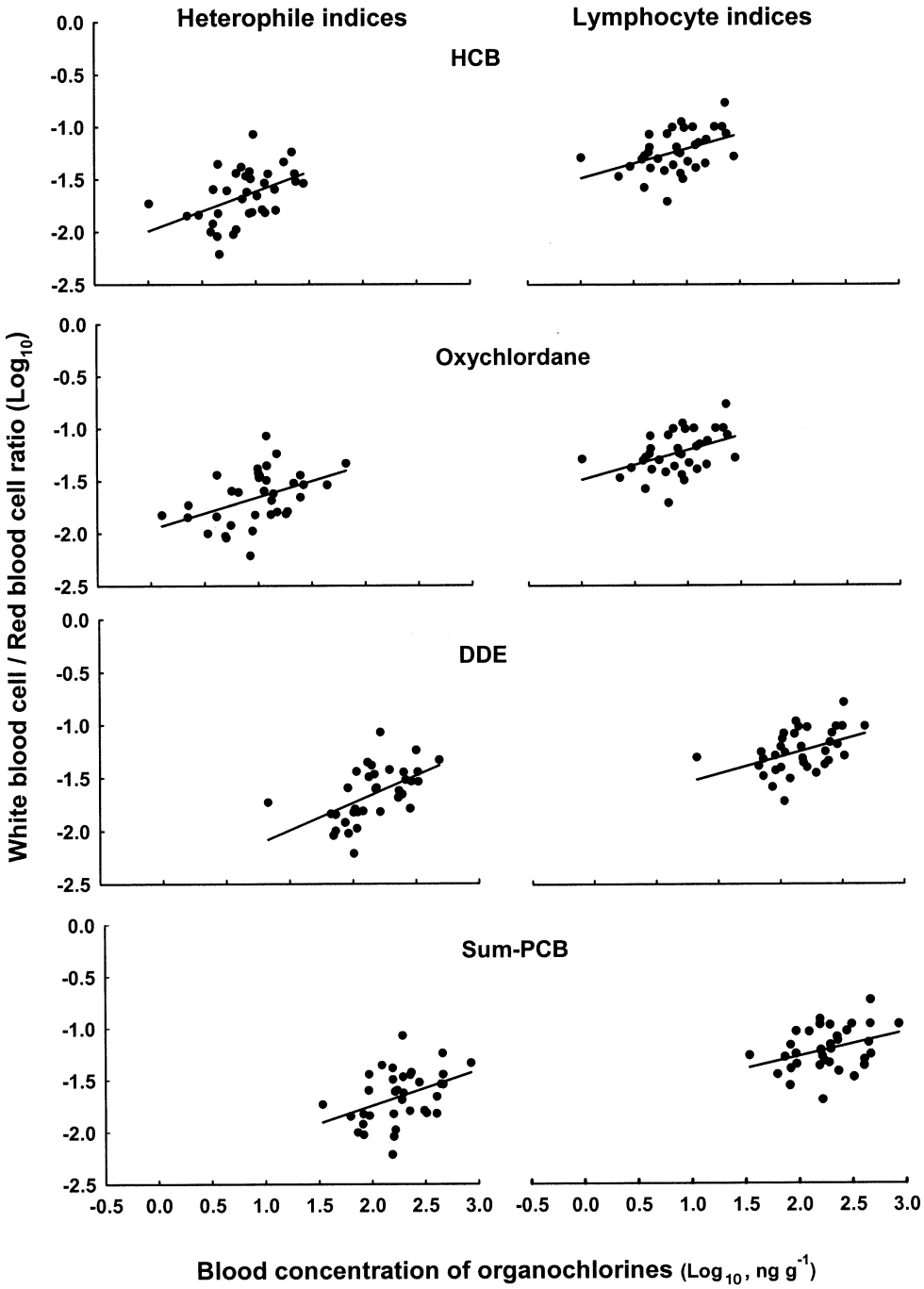

Fig. 1. The relationship between heterophil and lymphocyte indices obtained from blood smears in relation to blood concentrations of four different OCs (HCB, oxychlordane, DDE, and sum PCBs) in female glaucous gulls in 1997. Statistics in Table 4. HCB = Hexachlorobenzene; OC = organochlorine contaminant; PCB = polychlorinated biphenyl

heterophil indices and concentrations of any of the OCs in female gulls (Table 5), but in male gulls heterophil indices showed positive and significant relationships with oxychlordane and all PCB congeners (Table 5 and Fig. 3). There were no significant relationships between lymphocyte indices and concentrations of any of the OCs in male or female birds in 2001 ($0.27 < p < 0.99$).

Immune Responses Against Diphtheria-Tetanus Vaccine

For tetanus antibody response, there were no interactions between sex and OC concentrations for any of the OCs analyzed when controlling for year ($0.17 < p < 0.99$). Sex had no effect on tetanus antibody response ($F_{1,21} = 0.35$, $p = 0.56$) when controlling for year. Concentrations of OCs were not signifi-

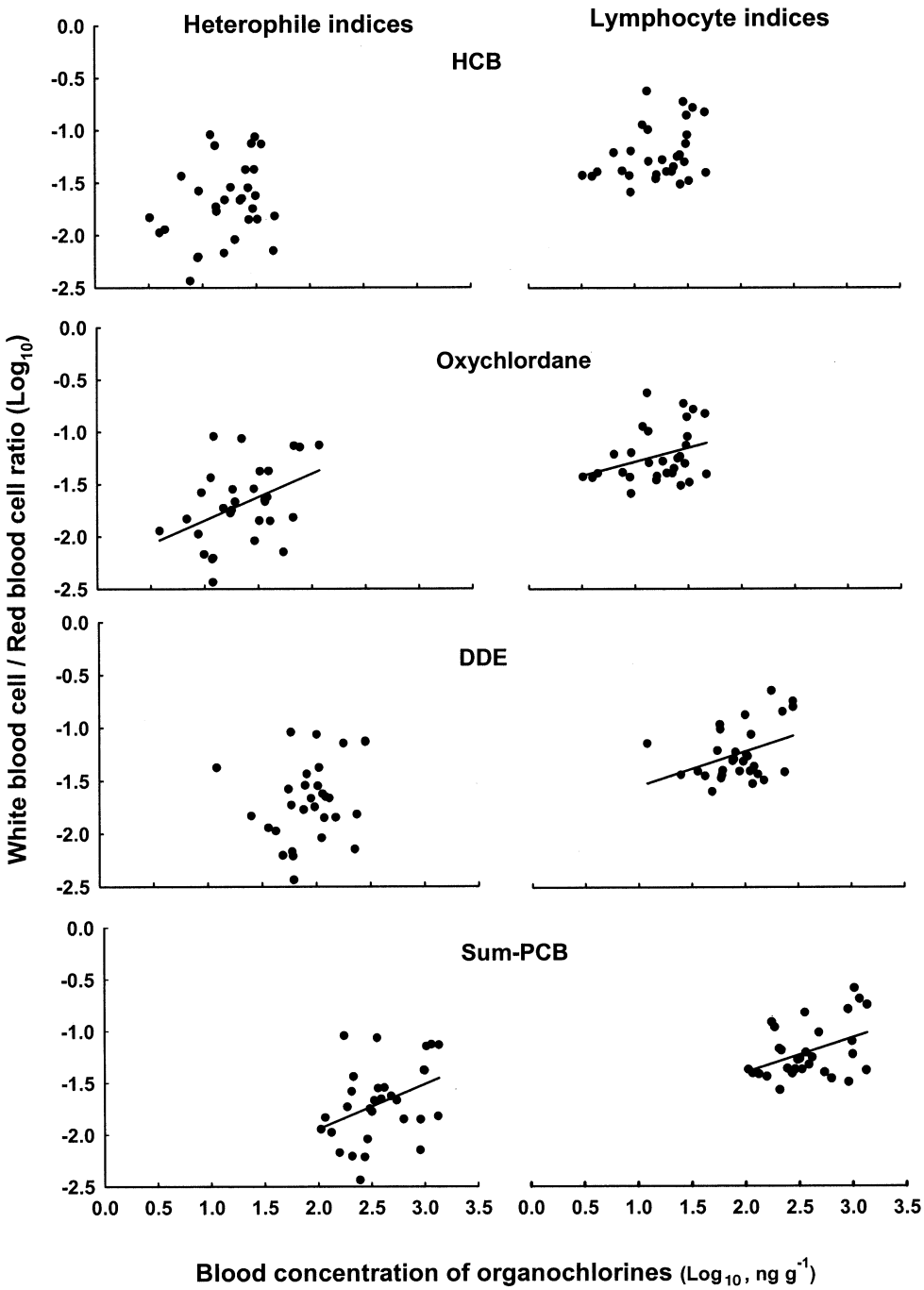

Fig. 2. The relationship between heterophil and lymphocyte indices obtained from blood smears in relation to blood concentrations of four different OCs (HCB, oxychlordane, DDE, and sum PCBs) in male glaucous gulls in 1997. Statistics in Table 4. HCB = Hexachlorobenzene; OC = organochlorine contaminant; PCB = polychlorinated biphenyl

cantly related to tetanus antibody response ($0.41 < p < 0.95$) when controlling for sex and year.

One outlier was removed for antibody response to diphtheria toxoid in female gulls (its value was 72.5 compared with a mean of 3.1, and the second largest value was 11.4). Year did not have any significant effect on the levels of antibody response to diphtheria toxoid in any analyses ($0.27 < p < 0.85$), but we controlled for this factor in all analyses. For HCB ($p = 0.03$) and oxychlordane ($p = 0.016$), there were significant interactions between OC level and sex, whereas for the other compounds, interactions were nearly significant ($0.05 < p < 0.1$) apart from DDE ($p = 0.72$) and PCB-118 ($p = 0.13$). This indicates that the two sexes responded differently to the diphtheria toxoid with respect to their levels of most OCs. We therefore analyzed the sexes separately. In female birds, when controlling for year, response against diphtheria toxoid was

Table 5. The relationship between the heterophil indices (log_{10} transformed) and blood concentrations of different OCs (log_{10} transformed) in glaucous gulls in 2001

	Female Gulls (N = 49)			Male Gulls (N = 51)		
Source	*p* Value	Trend	R^2	*p* Value	Trend	R^2
Heterophile indices						
HCB	0.33			0.45		
Oxychlordane	0.51			0.0097	+	0.129
DDE	0.64			0.13		
PCB-101	0.25			0.0585	+	0.071
PCB-99	0.29			0.0035	+	0.161
PCB-118	0.25			0.0139	+	0.117
PCB-153	0.30			0.0021	+	0.177
PCB-138	0.39			0.0073	+	0.138
PCB-180	0.29			0.0015	+	0.189
PCB-170	0.39			0.0017	+	0.183
Sum PCB	0.28			0.0028	+	0.168

HCB = Hexachlorobenzene.
OC = Organochlorine contaminant.
PCB = Polychlorinated biphenyl.

significantly and negatively related to HCB ($p < 0.01$; Table 6 and Fig. 4) and oxychlordane levels ($p < 0.05$; Table 6 and Fig. 4), but relationships to other compounds were not significant (Table 6). In males birds, no OCs showed significant relationships to antibody responses of diphtheria toxoid (Table 6).

Discussion

We found positive relationships between concentrations of different OCs and levels of WBCs. Similar studies of herring gulls (*Larus argentatus*) and young Caspian terns (*Sterna caspia*) from the Great Lakes in North America have also reported positive relationships between levels of WBCs, both heterophils and lymphocytes, and OCs (Grasman *et al.* 1996, 2000). These studies suggested that high organochlorine burdens in general result in more resources being allocated to proliferation of WBCs. The hepatic PCB and DDE levels in glaucous gulls at Bear Island were 3.5 and 1.2 ppm, respectively (Henriksen *et al.* 2000), which in general is lower than the levels in herring gulls in the Great Lakes (PCB range among colonies = 1.8 to 23.8 ppm and DDE = 0.6 to 7.4 ppm; [Grasmann *et al.* 2000]). This may suggest that relatively low OC levels may result in immune system effects in glaucous gulls.

A major problem in ecotoxicology is that free-living animals are exposed to mixtures of OCs of which the different components are highly correlated. Hence, elucidating what OCs actually cause effects is difficult (Jones and Voogt 1999; Bustnes *et al.* 2003). Moreover, effects may also be caused by subgroups of OCs (e.g., Jones and Voogt 1999). It is therefore important to be cautious when interpreting results obtained from different statistic tests run on the same samples because a significant result of one OC may only be a result high correlations with the OCs that are actually causing effects. However, if some compounds keep coming out as the strongest predictors of different effects, it may suggest that they are more important in causing adverse effects than others.

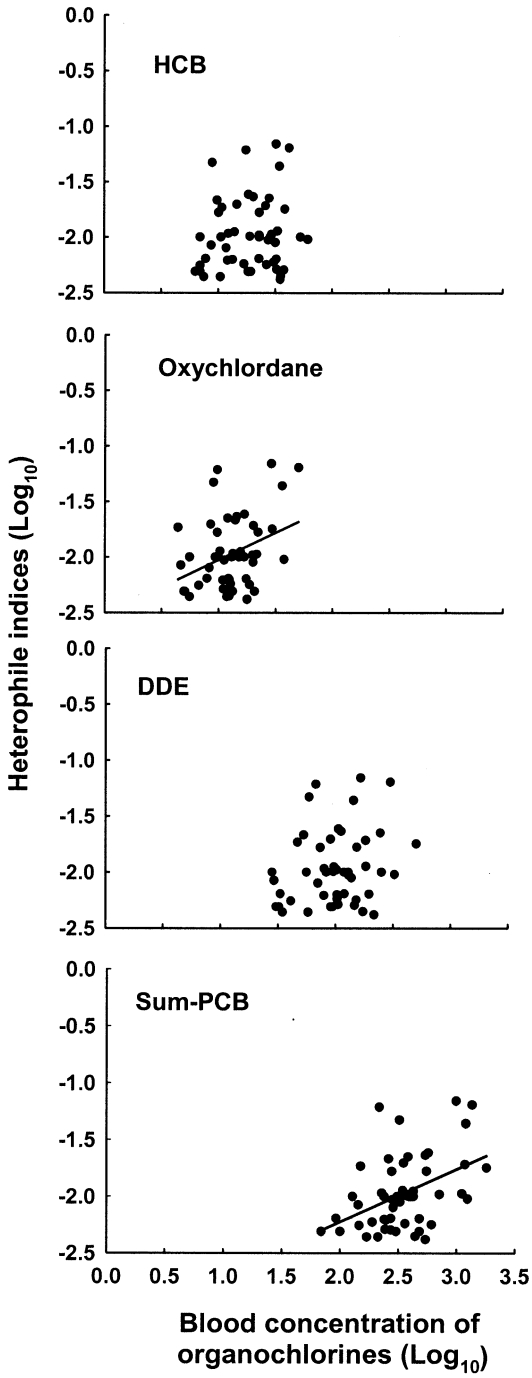

Fig. 3. The relationship between heterophil indices obtained from blood smears in relation to blood concentrations of four different OCs (HCB, oxychlordane, DDE, and sum PCBs) in male glaucous gulls in 2001. Statistics in Table 5. HCB = Hexachlorobenzene; OC = organochlorine contaminant; PCB = polychlorinated biphenyl

In glaucous gulls, all persistent OCs and heterophile indices were positively related for both sexes in 1997 and for male birds in 2001. Heterophils, which are the avian counterpart of

Table 6. The relationship between antibody response (mOD/min, rank transformed) to injection of diphtheria toxoid and blood concentrations of different OCs (log_{10} transformed) in glaucous gulls after controlling for year (2000 and 2001)

	Female Gulls (N = 13)			Male Gulls (N = 11)		
Source	*p* Value	Trend	r_s	*p* Value	Trend	r_s
HCB	0.0077	–	0.525	0.57		
Oxychlordane	0.0087	–	0.449	0.27		
DDE	0.80			0.47		
PCB-99	0.28			0.16		
PCB-118	0.30			0.23		
PCB-153	0.27			0.16		
PCB-138	0.26			0.13		
PCB-180	0.25			0.19		
PCB-170	0.30			0.16		
Sum-PCB	0.27			0.16		

HCB = Hexachlorobenzene.
mOD = Mean optical density.
OC = Organochlorine contaminant.
PCB = Polychlorinated biphenyl.

mammalian neutrophils, are phagocytosing cells that enter the tissues during inflammatory response (Parslow 1994; Ots *et al.* 1998). They are part of the innate immune defense system and play an important role during the initial stages of most infections. Heterophils are the primary means of controlling bacterial infections (Roitt *et al.* 1998). Inflammation, the nonspecific response to foreign invasion or tissue damage, is characterized by increased levels of heterophils (e.g. Dein 1986; Ots *et al.* 1998). Similarly, there were significant positive relationships between various persistent OCs and lymphocytes in both sexes in 1997. Lymphocytes (B and T cells) are the main cell types in the adaptive immune response. They are highly specific and will only proliferate once a specific antigen is recognized (Janeway *et al.* 1999). There are at least two explanations for the positive relationships observed in this study. Either (1) high concentrations of OCs are decreasing the function of immune cells, thus resulting in an increased production of immune cells to cope with low level infections or (2) OC-mediated immunosuppression may be contributing to infections and causing a compensatory increase in numbers of different WBCs but not in function (Grasman *et al.* 2000). The latter explanation seems more in accordance with the finding that birds with high OC levels tended to be more highly infected by nematode parasites in our study population (Sagerup *et al.* 2000).

The difference in relationships between OCs and WBCs between sexes is difficult to explain. However, there are a number of sex-related differences in hormone profiles and reproductive investment that may be influenced by OCs, which again may influence immune status (e.g., Vos and Luster 1989; Colborn *et al.* 1993; Sheldon and Verhulst 1996). Because male gulls had higher levels of OCs than female gulls, their immune system may be more affected, and—as expected—we found more correlations between WBC levels and OCs in male than female birds. Moreover, in 2001, male gulls had generally lower levels of OCs compared with levels in 1997 and at the same time fewer correlations between OCs and WBC indices. In addition, no OC was related to antibody responses against the combined diphtheria and tetanus toxoids in male gulls, suggesting that the humoral immune response was not affected in male birds. A prediction would be that in female birds, the relations between OC and WBCs in 2001 would be the same as in 1997 because female gulls had similar levels of most OCs in both years. However, there were no significant relations in 2001, a result for which we have no explanation.

Despite the absence of significant relations between WBC indices and OC levels in 2001, we found evidence for decreased immune responses against diphtheria toxoid in female gulls with high HCB (or oxychlordane) levels. Decreased ability to mount a specific immune response may be one proximate explanation for the higher parasite levels in birds with increased OC levels (Sagerup *et al.* 2000). However, Sagerup *et al.* (2000) found the strongest relations for heavily chlorinated PCBs, not for HCB or oxychlordane.

The differences in responses against diphtheria and tetanus may be explained by the findings in blue tits (*Parus caeruleus*), in which the tetanus response compared with the diptheria response, seems to depend on genetic background (it has a very high heritability), the diptheria response seems more condition dependent (Råberg and Stjernman 2003).

PCBs are known to have immunotoxic effects in gulls (e.g., Grasman and Fox 2001) and in glaucous gulls PCBs make up nearly 75% of the OCs we measured (Bustnes *et al.* 2003). It is thus very likely that they are important in causing immune stress in this species as well. Several PCB congeners were also strongly linked to increased WBC indices. However, the only changes in levels of PCB congeners between 1997 and 2001 were relatively small changes in PCB-99 and PCB-118 in male birds, two congeners that were not among those most strongly associated with WBC indices. Only HCB and oxychlordane levels changed strongly between the years. In female birds, HCB levels were higher in 2001 than in 1997. In male birds, oxychlordane levels were much lower in 2001 than 1997, whereas PCB-99 and PCB-118 showed a more moderate decrease between 1997 and 2001 in male gulls. In female gulls in 1997, HCB and DDE levels were strongly related to heterphil indices, and they were also the only compounds that were significantly related to lymphocyte indices, whereas HCB was related to the response to diphtheria toxoid in female birds. Recent studies of rats and humans have found that HCB interferes with different immune functions, but the compound seems to induce immunopathology through very complex mechanisms, probably involving multiple factors (Michielsen *et al.* 1999a, 1999b; Daniel *et al.* 2001). It is thus not possible at this stage to say definitively that HCB is the prime agent in suppressing immune functions in glaucous gulls. In male gulls, oxychlordane was the compound most strongly related to both heterophile and lymphocyte indices in 1997, and it was also related to heterophils in 2001 as were all PCB congeners. Oxychlordane is a metabolite from *cis*- and *trans*-chlordane, which are major compounds in chlordane, a cyclodiene insecticide previously used commonly in agriculture (Wiemeyer 1996). We are aware of no study that has demonstrated immune toxicity of oxychlordane, and the relationships may thus be simply a result of covariance with other OCs. However, studies have demonstrated the immune-modulating effects of chlordane, including impairment of macrophage function (Theus *et al.* 1992; Blaylock and Mehendale 1995). It is also interesting to note that in our study population, HCB levels were most strongly linked to reproductive effects (Bustnes *et*

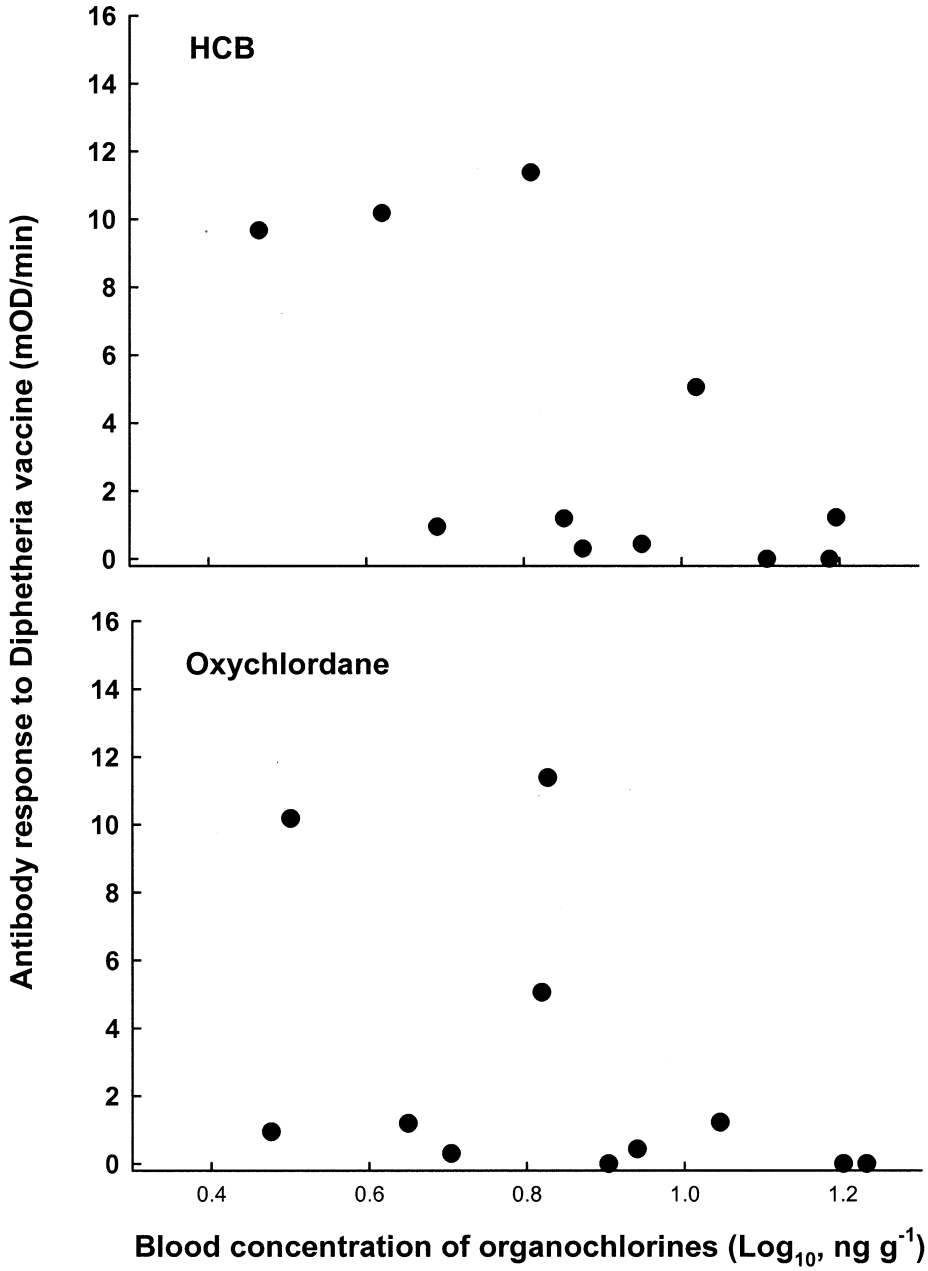

Fig. 4. Antibody response to injection of diphtheria vaccine (mOD/min) in relation to blood concentrations of HCB and oxychlordane in female glaucous gulls. Data from 2000 and 2001. Statistics in Table 6. HCB = Hexachlorobenzene, mOD = mean optical density

al. 2003) and fluctuating asymmetry in wing feathers (Bustnes *et al.* 2002), whereas oxychlordane has been found to be most strongly related to annual survival of adults (Bustnes *et al.* 2003).

In conclusion, this study provided indications that organochlorine pollution is influencing the immunocompetence of glaucous gulls, even in a pristine arctic area. Immune toxicity may therefore be one of the explanations for decreased probability of survival among birds with high OC concentrations (Bustnes *et al.* 2003). However, before such a conclusion can be drawn, additional research is needed to elucidate the causal relationships between immune function, disease, and organochlorines.

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