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IMMUNOSUPPRESSION IN THE NORTHERN LEOPARD FROG (*RANA PIPIENS*) INDUCED BY PESTICIDE EXPOSURE

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Abstract—An injection study and a field study were used to investigate the hypothesis that environmental xenobiotics have the potential to alter the immune function of northern leopard frogs (*Rana pipiens*). Three assays, IgM-specific antibody response to keyhole limpet hemocyanin linked to dinitrophenyl (KLH-DNP), zymozan induced chemiluminescence (CL) of whole blood and the delayed-type hypersensitivity (DTH), were used to assay humoral, innate and cell-mediated immune endpoints. Sublethal doses of DDT (923 ng/g wet wt), malathion (990 ng/g wet wt), and dieldrin (50 ng/g wet wt) were used in the injection study. In all pesticide-injected groups, antibody response was dramatically suppressed, DTH reactions were enhanced, and respiratory burst was lower. When the order of administration of pesticides and antigens was reversed, no differences in immune function between the control and dosed groups were apparent, indicating that frogs exposed to pathogens prior to pesticide exposure can still respond. A field study found significant differences in immune function between frog populations in pesticide-exposed and pesticide-free locations. The antibody response and CL were suppressed and the DTH enhanced in frogs from Essex County (ON, Canada). Overall, the results suggest that exposure to these pesticides can cause both stimulatory and suppressive immune changes in adult frogs and is doing so in wild populations.

Keywords—Frog Immunosuppression Pesticide Antibody DDT

INTRODUCTION

Amphibian populations are in decline worldwide [1–3], and in a number of these cases, infectious disease has been the proximal cause of death [4]. Local declines and extirpations of amphibian populations have been associated with pathogenic infections, especially fungal and viral pathogens [5] that are ubiquitous in freshwater. While immunosuppressed individuals are reportedly more susceptible [6] and no linkage between immunosuppression and emerging infectious diseases has been shown, this may be an important aspect of amphibian declines. Though many hypotheses have been proposed to account for population declines and disappearances, in most cases, the cause or causes are still uncertain [1,7].

Many chemicals introduced into the environment by human activity have the capacity to disturb the immune system of wildlife and humans. Experimental studies demonstrate that exposure to synthetic chemicals can result in increases or decreases in measured immune parameters and hypersensitivity [8]. A number of organochlorine pesticides, such as DDT and dieldrin, and organophosphate pesticides, such as malathion, have the potential to produce immunotoxicity in a variety of species [8]. Experimental results are consistent with wildlife studies that have demonstrated contaminant-induced immunosuppression. Numerous studies quantify the levels of different compounds in amphibians but relatively few address the sublethal effects. It is plausible that pesticide exposure may adversely affect the immune function of exposed amphibian populations, making them more susceptible to a variety of infections.

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Organochlorine pesticide use in North America has decreased since the 1970s, but pesticides still pose a threat to biota because of their toxicity, environmental persistence, and potential to bioaccumulate in food chains [9]. The historic application of DDT to wetlands for mosquito control may be of great importance in affecting amphibian populations [10]. Essex County is one of the premier agricultural areas of Canada. A wide variety of cash crops (i.e., tomatoes, strawberries, lettuce, fruit, etc.) are grown, as well as wheat, corn, and soybeans. This agricultural use means that this area is subject to large amounts of pesticide use. The ecology and physiology of amphibians may expose them to a wide variety of routes of contamination by these compounds [11]. Both DDT and dieldrin have recently been found in the tissues of frogs from Point Pelee [9] at concentrations up to 160 and 199 μg/kg, respectively. Organochlorine insecticides are known to significantly alter the function of the immune system [12].

A recent study examining pond communities in southwestern Ontario included anecdotal reports of *Rana pipiens*, the most abundant frog in the Essex plain, declining during the study period, 1992 to 1993 [13]. Since 1972, three species of amphibians have become locally extinct in Essex county and a fourth species has been reduced in abundance in Point Pelee National Park [9]. The fact that leopard frogs have not been seen in Point Pelee National Park for several years, combined with the observation that high levels of DDT were present in the park, were part of the impetus to initiate this project.

The immune system plays a crucial role in maintaining health. Amphibian immune defenses involve both innate defense mechanisms (i.e., those present before the pathogen is introduced) and adaptive components that respond specifically

to each pathogen. When these two arms of the immune system are taken together, frogs lack only a few elements of mammalian immune systems [4]. The innate immune system provides rapid, nonspecific protection until the adaptive immune response can be mobilized while also providing signals that initiate and shape the adaptive response [14]. Examination of the innate immune system can be achieved by investigating phagocytic pathways. Amphibians possess phagocytic cells, such as macrophages and neutrophils, that are capable of directly engulfing a pathogen [4,15]. Oxidative burst in phagocytes is an indication of the activation of killing mechanisms [16] and can be examined using a chemiluminescent technique. Phagocyte activation results in the release of a variety of reactive oxygen compounds by the nicotinamide adenine dinucleotide phosphate reduced-form oxidase complex and myeloperoxide [17]. These processes generate electronically excited states, which, on relaxation to the ground state, emit photons. This can be measured from a whole-blood sample using a luminometer provided that the reaction is amplified by the addition of luminol and by using zymozan to induce the oxygen radical production. When used with whole blood, this assay measures oxygen radical production by circulating neutrophils, an important aspect of innate immunity.

Specific immune responses include both humoral immunity and cell-meditated responses. Humoral immunity occurs following the introduction of foreign macromolecules and the subsequent production of antigen-specific antibodies by plasma cells. Antibody titers in serum can be measured by ELISA to assess the strength of a humoral response. The keyhole limpet hemocyanin linked to dinitrophenyl (KLH-DNP) is a strong protein antigen that can induce either humoral or cellular immune responses [16] and is therefore an ideal candidate for assessing loss of antibody reactivity. Cell-mediated immunity is the second arm of the specific immune response and involves T-lymphocytes and, to a lesser extent, macrophages and polymorphonuclear leukocytes. A good correlate of cell-meditated immunity is the delayed-type hypersensitivity (DTH) reaction. The assay requires the specific recognition of a given antigen by activated T-lymphocytes, which subsequently proliferate and release cytokines. These cytokines increase vascular permeability, induce vasodilatation, and cause macrophage accumulation [16]. These processes result in a localized swelling that can be measured with fine calipers.

This study investigated whether pesticides caused measurable alterations in the immune function of amphibians. The immunological methods developed were nonlethal, allowing for repetitive sampling of animals. To date, there have been few studies assessing the health of pesticide-exposed amphibian populations by examining immune function in a nonsacrificial manner. An injection study, in which the order of antigen and pesticide administration were reversed, and a field study were carried out. The aim of the injection study was to determine whether a sublethal dose of a known immunosuppressant pesticide would elicit immune alterations in adult frogs. The study in which test antigens were administered before the pesticides determined if pesticides destroy immune function or simply inhibit it as well as investigating whether individuals could mount an immune response if they were immunized prior to pesticide exposure. For both laboratory studies, the effects of pesticides were compared with cyclophosphamide, an immunosuppressive drug. Finally, the field study applied the laboratory-developed techniques to northern leopard frogs collected from different areas of Ontario to determine whether spatial variation in immune function existed. Organochlorine contaminant levels in whole-frog homogenates were evaluated to establish whether alterations in immune function could be correlated with contaminant levels.

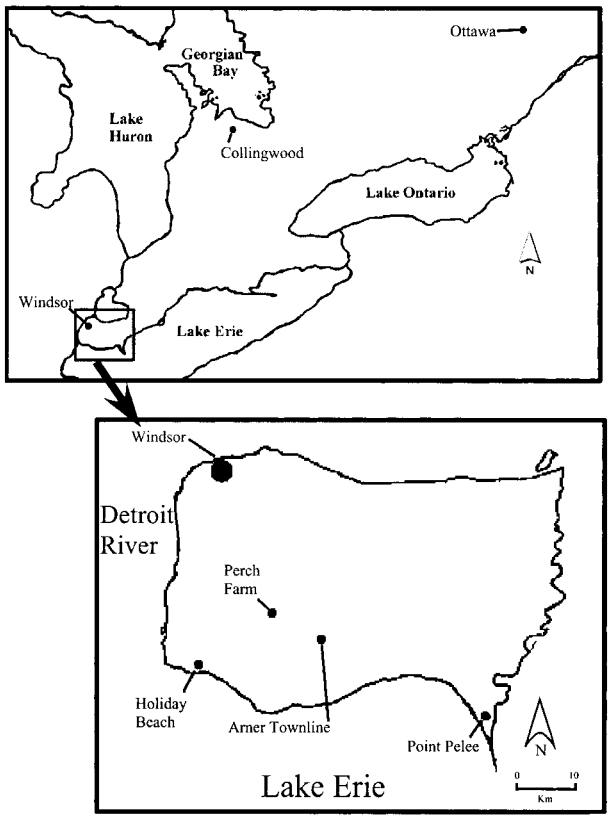

Fig. 1. Collection sites in Ontario, Canada, for *Rana pipiens* in the autumn of 2000. The inset shows where the frogs were collected in Essex county.

MATERIALS AND METHODS

Frog husbandry

Northern leopard frogs (*Rana pipiens*) used in the injection study were captured from the Collingwood field sites in Ontario in 1999 and 2000 and were kept in the lab for at least eight weeks before the experiments were initiated. The frogs used in the field study were collected from the sites shown in Figure 1, which included two control sites and four sites in Essex county. All animals were maintained in the aquatic facility at the Great Lakes Institute for Environmental Research at the University of Windsor (Windsor, ON, Canada). The frogs were housed in aquaria (30 × 90 cm) in groups of five and provided with a washed concrete block covered in rubber matting as a feeding platform. Underwater charcoal filters (QuickFilter® [802], Hagen, Montreal, QC, Canada) were utilized to remove waste products from the water. The tanks were emptied and scrubbed once a week and refilled with chlorinated tap water. Frogs were fed daily with crickets that were maintained on a diet of bird granules (Tropican, Hagen). Individuals were identified using a combination of close-up photographs and line drawings of markings accompanied by a detailed written description. This study was performed in accordance with protocols set forth and under full approval of the Animal Care Council of Canada (Windsor, ON, Canada).

Immunization

A stock solution containing 2 mg/ml of keyhole limpet hemocyanin linked to DNP (Calbiochem, La Jolla, CA, USA), 2 mg/ml phytohemagglutinin (PHA-P; Sigma-Aldrich, St. Louis, MO, USA), and 16 mg/ml of dimethyldioctadecylammonium bromide (Fluka Chemika, Ronkonkoma, NY, USA) was prepared. Titermax Gold (Sigma-Aldrich) was used as the adjuvant at a ratio of 2.5 ml:1 solution:adjuvant. Each frog received 100 μl of this emulsion intramuscularly in the left thigh.

The injection-study frogs were immunized 2 d after exposure to the relevant pesticide. To determine the importance of the immunization order, three groups of frogs were immunized 5 d prior to pesticide exposure. The field-study frogs were immunized within the first month of capture. For repeated measurements of the antibody response to KLH-DNP, individual frogs were boosted with the above mixture two weeks prior to blood collection.

Blood sampling

Blood was collected by cardiac puncture using a 28-gauge needle and a 1-cm^3 syringe. The anticoagulant used was lithium heparin (10,000 units/ml deionized water; Sigma-Aldrich). During the procedure, individual frogs were immobilized by wrapping in damp paper towels. After collection, blood was stored in a 1.5-ml microcentrifuge tube (Diamed, Mississauga, ON, Canada) and either stored whole at 4°C until used for chemiluminescence analysis (within 10 h) or allowed to clot, after which the serum was stored at 4°C until ELISA analysis (24–48 h).

Antibody response to KLH-DNP

Microtiter plates (96 well) were precoated overnight with KLH in coating buffer (15 mM Na_2CO_3, 34.88 mM $NaHCO_3$, 3.125 mM NaN_3, pH 9.6) and incubated at room temperature. The KLH solution was retained, stored at 4°C, and reused 10 times. The plate was washed three times using tris buffered saline solution ([TBS-T]: 136.89 mM NaCl, 2.68 mM KCl, 24.76 mM tris base, 0.5 ml/L Tween, pH 8) in a squeeze bottle. Three hundred microliters of blocking buffer (5% gelatin in TBS-T) was added to all wells and the plate was incubated for 1 h at 37°C. The plate was washed three times using TBS-T. The blood samples were centrifuged (2,000 rpm for 5 min at 4°C) in 1-ml Eppendorf® (Westbury, NY, USA) tubes and the plasma removed. A 100-μl aliquot of plasma was placed into appropriate wells in replicates of four. The plate was washed three times with TBS-T. The primary antibody used was 6 to 16 [18], a mouse monoclonal antibody that was raised against *Xenopus* anti-IgM. One hundred microliters of antibody solution was added undiluted to all wells and incubated at 37°C for 1.5 h. The plate was again washed three times with TBS-T, followed by addition of 100 μl of secondary antibody solution (1:1,000 in TBS-T), and the plate incubated for 1 h at 37°C. The secondary antibody was antimouse IgG (whole molecule) alkaline phosphatase conjugate, developed in goat (Sigma-Aldrich). The plate was washed three times with TBS-T and 50 μl of alkaline phosphatase developing solution (Sigma Fast *p*-nitrophenyl phosphate tablet set, Sigma-Aldrich) added to all wells. After 30 min, 50 μl of 0.03 M NaOH was added as a stop solution. Three control wells were set up in replicates of four on each plate, one that contained fetal calf serum (Invitrogen Life Technologies, Burlington, ON, Canada) in place of frog plasma and the other two wells containing primary and secondary antibody only. Results were read on a plate reader (MRX II, Dynex Technologies, Chantilly, VA, USA) at 405 nm.

Delayed-type hypersensitivity skin response to PHA-P

Nine days after immunization, a 2-mg/ml solution of phytohemagglutinin P (PHA-P) in PBS was prepared. The thickness of the middle toe of the right and left hind foot at the point where the webbing ends was measured using an electronic micrometer (Digimatic Outside Micrometer 0-1″, Mitutoyo, Morgan Precision Tools, Aurora, IL, USA). The right toe was injected (0.5 cm^3 Micro-Fine insulin syringe, 0.33 × 13 mm/29 gauge × one half, Becton Dickinson, NJ, USA) with the PHA-P solution and the left toe was injected with the PBS to determine the nonspecific inflammation. Measurements of both sites were taken 24, 48, and 72 h after injection. The area to be measured was extremely small, and the sensitivity and accuracy of this method was determined in a double-blind measurement study using unexposed frogs in a 4-d trial.

Chemiluminescence

The zymozan-induced chemiluminescence of whole blood was determined using the method developed by Marnila et al. [17]. The assay was performed on a 96-well microtiter plate in replicates of four within 10 h of the blood sample being taken. A replicate of blanks wells was included on each plate. Each well contained whole blood (500 μl diluted in 200 μl of frog Ringer's solution [116 mM NaCl, 1.2 mM KCl, 1 mM $CaCl_2$, 2.70 mM $NaHCO_3$], and 1 mM Luminol [5-amino-2,3-dihydro1,4-phthalazinedione]; Sigma-Aldrich). The contents of the four wells were made up in an Eppendorf, vortexed lightly, and a 225-μl aliquot added to each well, and each plate was incubated for at least 10 min before reading. Directly prior to reading the plate, 500 μg of Zymozan (Zymosan A, Sigma-Aldrich) was added. Each well had a total volume of 250 μl. The chemiluminescent emissions were measured at 3-min intervals for 30 readings to obtain kinetic curves on a luminometer (MLX Microtiter Plate Luminometer, Dynex Technologies, Denkendorf, Germany). Results were presented as relative light units. The peak chemiluminescence was used as the sample value.

Experimental design

In the injection study, dosing was administered by a subcutaneous injection of DDT, dieldrin, malathion, or cyclophosphamide (Cy). It was not necessary to undertake range finding of dosages in frogs because sublethal doses have been published; e.g., DDT and dieldrin doses for *R. pipiens* have been reviewed by Harfenist et al. [19]. Sublethal malathion doses were taken from a study using Woodhouse's toads [20], and the dosage for Cy was determined for *R. pipiens* by Bugbee et al. [21], although, for this compound, a lower dose was used than the earlier study. Stock solutions of 250 mg/L *o′p*,DDT, (Sigma-Aldrich), 25 mg/L dieldrin (Sigma-Aldrich), 330 mg/L of technical-grade malathion (White Rose, Windsor, ON, Canada), and 3,000 mg/L Cy (Sigma-Aldrich) were made up in DMSO. Each group of frogs (n = 11) was exposed to specific compounds by giving each individual in the group an intramuscular injection in the right thigh with 3 μl/g body weight of the appropriate pesticide or control solution. The control group received a 3 μl/g body weight injection of DMSO. One frog from each group was sacrificed after 2 d for

analysis of the actual contaminant dose delivered. The remaining 10 frogs in each group, while maintained in a single tank, were split into two subgroups of five, one group of which was used for the antibody response and DTH assays while the other was used for the chemiluminescence assay.

An experiment in which the order of administration of pesticides and test antigens was reversed was undertaken using three exposure groups containing four frogs per group. Stock solutions used were 25 mg DDT in DMSO, 300 mg/L Cy in DMSO, and DMSO-only control group. Each group of frogs was exposed to the appropriate solution by an intramuscular injection in the right thigh with 3 μl/g body weight of each pesticide solution.

For the field study, frogs were collected from sites in southern Ontario, Canada, as shown in Figure 1. The control sites were an equine facility near Ottawa and a marsh near Hepworth, north of Collingwood. The sites in Essex County were a wooded lot near Arner Townline, a creek just north of Point Pelee, a perch farm near McGregor (that had not been sprayed with pesticides in recent years), and a wetland area adjacent to Holiday Beach. Thirteen to 15 frogs were collected at each site; 10 were used to determine immunological competence and the remaining frogs were sacrificed for contaminant analysis. After capture, the frogs were acclimated in captivity for approximately one to three weeks to recover from transport stress. Collections from all sites were finished prior to the initiation of immune assays. Immunological assays were undertaken within the first four weeks of capture and repeated two months later.

Contaminant analysis

For the injection study, frogs were sacrificed at 2 d and 20 weeks after exposure to evaluate retention of the injected dose during the study duration. For the field study, two to five frogs per site were sacrificed for contaminant analysis upon arrival at the laboratory and stored frozen (−20°C) in hexane-rinsed foil. Sample preparation was done using an adaptation of the method described by Lazar [22]. Briefly, whole frogs were homogenized and stored in chemically clean glassware. Carcass homogenates (1–2 g) were ground with 20 g Na_2SO_4. The homogenate was wet packed in a 2.1-cm i.d. × 24-cm glass column containing 50 ml dichloromethane:hexane (1:1 v/v) and spiked with 12.5 ng of 1,3,5-tribromobenzene (Accu Standards, New Haven, CT, USA) for use as a surrogate standard. After 1 h, the solvent was eluted from the column and the homogenates extracted with a further 250 ml dichloromethane: hexane (50:50% v/v). The extracts were evaporated to 10 ml. A 10% aliquot was removed for lipid determination by gravimetric analysis and the remaining extracts cleaned by gel permeation chromatography [23]. Further clean-up of concentrated extracts were performed by Florisil chromatography. Extracts were loaded onto a 1-cm i.d. × 24-cm glass column that was wet packed with 6 g activated Florisil (BDH, Toronto, ON, Canada) in hexane with a 1-cm Na_2SO_4 cap. Organochlorine chemicals were eluted from the Florosil with 50 ml hexane, followed by 50 ml of dichloromenthane:hexane (15: 80% v/v) followed by elution with 130 ml of dichloromenthane:hexane (60:40% v/v).

Gas chromatographic (GC) analysis was performed on a Hewlett-Packard (Avondale, PA, USA) HP-5890 GC equipped with a 63 Ni electron-capture detector and HP-7673 autosampler. The column was a 30-m DB-5 fused silica capillary column (0.25-μm film thickness; J&W Scientific, Rancho Cordorva, CA, USA). Organochlorines were identified by retention time, and quantification of individual compounds was based on the response factors from a well-characterized standard mixture [24]. Standards were injected for every five samples analyzed and duplicates were added after the sixth sample.

Statistical treatment

All statistics were performed using Systat version 7.0. Differences among groups were determined using analysis of variance (ANOVA); a Tukey's multiple comparison test was used to establish which of the groups were distinct from each other. Outliers were omitted according to Studentized residual values generated by Systat.

RESULTS

Pesticides and humoral immunity: Antibody response to KLH-DNP

The antibody response to KLH during the pesticide injection study is presented in Figure 2A. To account for variation due the fact that an assay was performed for each time point independently every two weeks over the course of the experiment (measured by the fetal calf serum control performed with each assay) and to isolate the effects of pesticides alone as compared with possible effects of treatment with DMSO, the results are expressed as a percentage of the KLH-specific antibody produced by the negative control frogs (DMSO exposed). Two weeks after exposure, the groups injected with DDT, malathion, and Cy (positive control) produced negligible quantities of specific antibody, only 1 to 2% of the amount produced by the DMSO-injected animals. The dieldrin-exposed frogs produced about 30% of the specific antibody produced by the DMSO control frogs two weeks after pesticide exposure. By week 4, the DDT-, malathion-, and Cy-exposed groups produced slightly higher mean KLH-specific antibody levels, about 20 to 35% of the amount produced by the positive control frogs. There was little change in the antibody response of the dieldrin-exposed frogs, however, which remained around 35% of the response of the DMSO control frogs. No further recovery of the DDT- and dieldrin-exposed animals was evident by week 8, with response levels that were 20 and 38% of the DMSO-injected frogs, respectively, although the malathion-exposed group still showed responses that were less than 4% of the negative control group response levels. The amount of KLH-specific IgM produced by both the pesticide and positive control groups was significantly lower ($p < 0.001$; ANOVA) than the DMSO control frogs at two, four, and eight weeks after exposure. By week 20, all pesticide-exposed groups had recovered the ability to produce antibody specific to KLH. The IgM levels produced by the Cy-exposed group, however, remained depressed, constituting only 56% of the amount produced by the DMSO control frogs ($p < 0.001$). Malathion-exposed frogs were capable of greater antibody response than the DMSO group (130%), and the values for DDT and dieldrin, 91 and 74% of control levels, respectively, did not differ significantly from the DMSO control group.

The loss of antibody production in response to the specific antigen KLH-DNP was not apparent when the frogs were immunized with KLH-DNP 5 d prior to pesticide exposure, as seen in Figure 2B. There were no statistical differences among all three groups of frogs ($p > 0.2$; ANOVA) at both two and four weeks after pesticide injection, indicating that pesticide exposure does not destroy immune capacity or interact directly

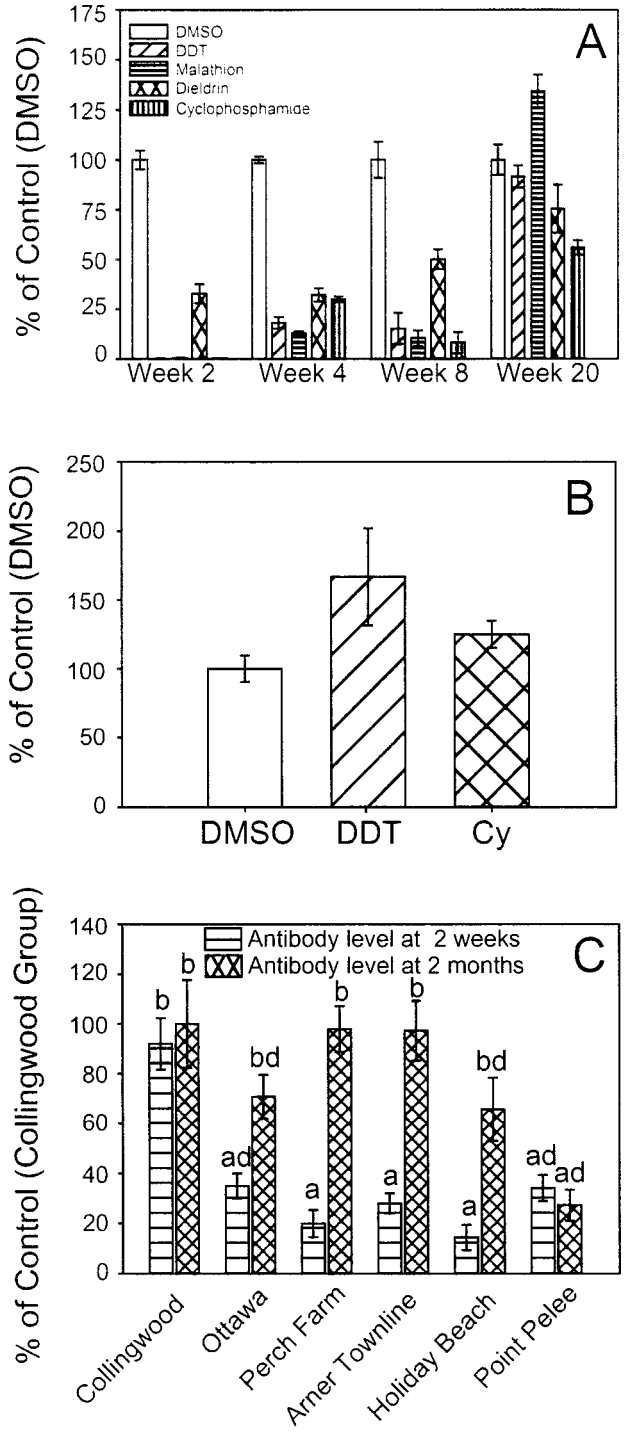

Fig. 2. (**A**) Anti-keyhole limpet hemocyanin (KLH)-IgM levels as determined by ELISA in serum of leopard frogs 2, 4, 8, and 20 weeks after exposure to DMSO, DDT, dieldrin, malathion, and cyclophosphamide (Cy); $n = 5$ per group ($p \leq 0.0001$). (**B**) Anti-KLH IgM levels as determined by ELISA two weeks after contaminant exposure for frogs immunized 5 d prior to contaminant exposure; $n = 4$ per group ($p = 0.122$). (**C**) Comparison of IgM antibody response to KLH within the first month and after two months in captivity in field-collected leopard frogs from regions of southern Ontario, Canada (near Ottawa [$n = 8$] and Collingwood [$n = 7$] and from the following areas of Essex County: perch farm ($n = 7$), Arner Townline ($n = 11$), Holiday Beach ($n = 12$), and Point Pelee ($n = 12$) ($p \leq 0.0001$).

to prevent antibodies from binding to antigens; it only inhibits immune responses when present prior to the antigen or pathogen.

In the field study, the antibody response to KLH-DNP was assayed two weeks after capture and differences were apparent among the groups collected from different regions of Ontario. Frogs collected near Collingwood showed a dramatically higher antibody response that was statistically different from other groups ($p < 0.001$; ANOVA); however, significant differences in antibody response were not observed among the other groups (Fig. 2C). When expressed as a percentage of the Collingwood group, antibody production was 35% for the Ottawa group, 20% for perch farm group, 28% for frogs from Arner Townline, 14% for the Holiday Beach group, and 34% for the Point Pelee frogs. Thus, populations from different locations exhibit clear differences in antibody-producing capabilities.

When the antibody response assay was repeated with the field-collected frogs two months later, the results were quite different (Fig. 2C). All the groups were statistically similar except for Point Pelee, which had an antibody response that was significantly lower than the other groups ($p < 0.001$; ANOVA). When expressed as a percentage of the response of the Collingwood group, the frogs from the Ottawa region were at 80% of control level, the Arner Townline group were at 97% of control, the perch farm group were at 98% of control, and the Holiday Beach frogs were slightly lower with 66% of control antibody production levels. The frogs from Point Pelee, on the other hand, were essentially unchanged at 27% of control levels, only 7% lower than their initial value. Thus, during the two-month laboratory captivity period, the frogs from all sites (except Point Pelee) had regained their ability to produce antibodies in response to a specific antigen.

Pesticides and cellular immunity: Delayed-type hypersensitivity to PHA-P

The DTH results from the injection study, as shown by the mean increase in toe thickness, is illustrated in Figure 3A. The results are plotted as the difference in thickness (24-h measurement minus initial measurement) of the right (PHA-injected) toe minus the left (PBS-injected) toe. All the pesticide-exposed frog groups had a higher response than the DMSO control frogs, although the results are were not significant ($p > 0.5$; ANOVA) due to the high variability.

When the order of immunization precedes pesticide exposure by 5 d, the DTH reaction showed a pattern opposite to the above injection study (Fig. 3B). The DMSO group showed a higher reaction than the DDT and Cy groups, although once again, there were no statistical differences ($p > 0.7$; ANOVA) due to the large degree of variation.

The DTH results in Figure 3C were obtained from the field-study frogs two weeks after capture. The frogs collected from Collingwood had the lowest DTH reaction, which was not statistically different from the Holiday Beach group ($p < 0.05$; ANOVA), but both groups' DTH reactions were statistically different from the relatively high reactivity of the Arner Townline group ($p < 0.05$; ANOVA). The other groups were not statistically different from either the high- or low-reacting groups. In general, frogs from the less contaminated Collingwood site showed a lower DTH reaction than those from sites that generally receive greater contaminant exposures, similar to the pattern observed in the injection study.

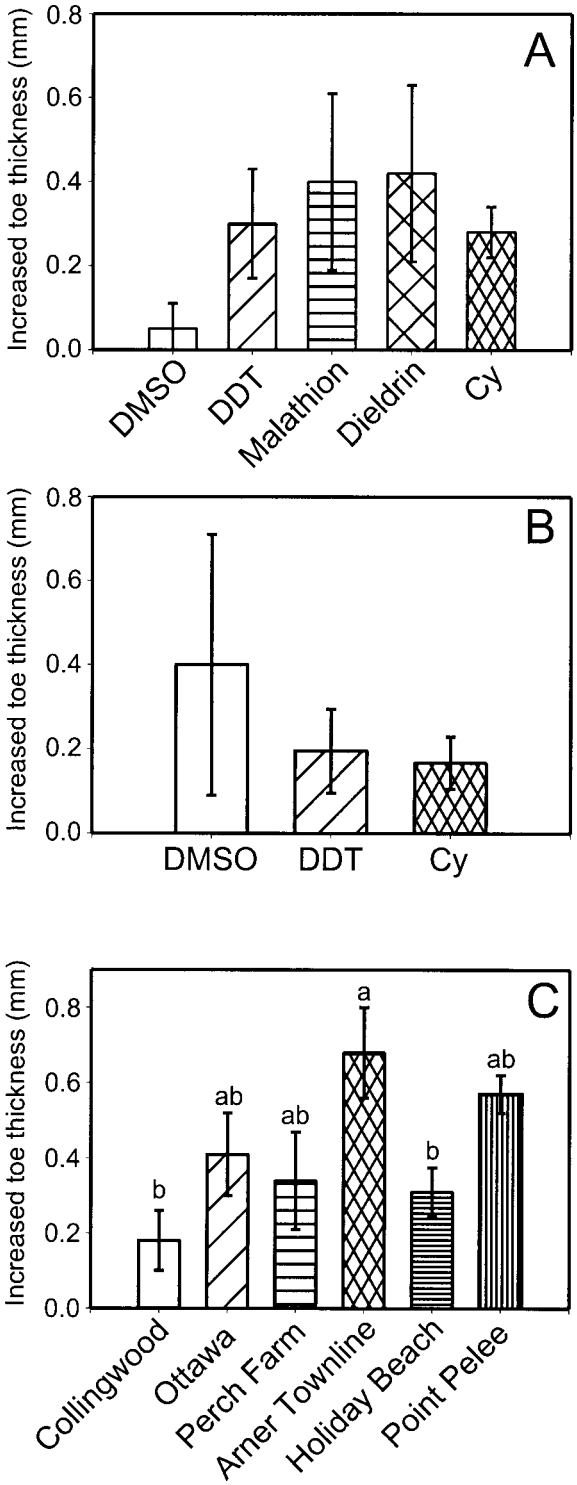

Fig. 3. (**A**) Delayed-type hypersensitivity (DTH) as determined by change in toe thickness in response to phytohemagglutinin (PHA) in leopard frogs two weeks after exposure to DMSO, DDT, dieldrin, malathion, and cyclophosphamide (Cy); $n = 5$ per group ($p = 0.597$). (**B**) The DTH reaction to phytohemagglutinin-P (PHA-P) measured two weeks after contaminant exposure for frogs immunized with PHA-P 5 d prior to contaminant exposure; $n = 4$ per group ($p = 0.743$) (**C**) Mean increase in toe thickness as an indication of DTH reaction in response to PHA-P at 24 h in field-collected leopard frogs from the regions near Ottawa ($n = 8$) and Collingwood ($n = 7$) and in Essex County, including the perch farm ($n = 6$), Arner Townline ($n = 11$), Holiday Beach ($n = 12$), and Pelee ($n = 12$) ($p = 0.005$). All study sites are located in Canada. Columns with the same lowercase letter have no statistical difference.

Pesticides and innate immunity: Oxidative burst products in whole blood

For the injection study, the mean peak chemiluminescence (CL) values for week 8 are presented in Figure 4A. The malathion and dieldrin frogs exhibited CL values that were significantly lower ($p < 0.05$ ANOVA) than the DMSO control or the DDT- or Cy-exposed frogs. This demonstrates that pesticides can also modulate cellular immune responses. In the reverse-order administration experiment, there were no statistical differences among the groups ($p > 0.05$; ANOVA) two weeks after immunization, although the Cy group had slightly higher CL values (Fig. 4B). Four weeks after immunization, the response of the Cy group and the DDT group was higher than the DMSO control group. The only statistical difference, however, was between the DMSO group and the Cy group ($p < 0.05$; ANOVA).

Figure 4C shows peak CL activity in field-collected frogs two weeks and two months following captivity. After two weeks, peak CL values showed differences ($p < 0.001$; ANOVA) among the sites. In the initial CL assay, mean peak CL values for Ottawa and Point Pelee frogs were higher than for other groups. The peak CL values for the Ottawa and Collingwood frogs were statistically similar, but the Collingwood group was significantly lower ($p < 0.001$; ANOVA) than the Point Pelee group. Arner Townline, Holiday Beach, and the perch farm frogs had peak CL values that were statistically lower ($p < 0.001$; ANOVA) than the Ottawa, Point Pelee, and Collingwood groups. As observed for both the KLH response and DTH assays, the CL assay was capable of discriminating spatial differences in immune function among southern Ontario frog populations. When the same frogs were assayed two months after the initial sampling period, the peak CL values for all groups except Holiday Beach and Point Pelee had not changed, although differences still existed among the groups ($p < 0.0001$). Overall, while the Point Pelee frogs lost oxygen radical-producing ability and the Holiday Beach frogs gained oxygen-burst capability, the other groups did not change much during the two months they were in the laboratory.

Contaminant analysis

During the injection study, whole-frog contaminant analysis was carried out on one individual from each exposure group on the second day after injection in order to confirm the dose that was received (Table 1). The expected concentration of DDT immediately after injection was 750 ng/g body weight, but the actual amount of DDT in the frog sacrificed 2 d after exposure was 923 ng/g. The levels of dieldrin were lower than the intended dose: The intended final concentration was 75 ng/g, but the actual concentration was 50 ng/g. Contaminant analysis was undertaken on five DDT-injected frogs and three dieldrin-injected frogs 20 weeks after contaminant exposure (Table 1). In each case, pesticide concentrations in treatment animals after 20 weeks of injection were increased by at least two orders of magnitude above ambient chemical concentrations in the laboratory population. Concentrations of DDT in the control and dieldrin-injected frogs averaged 0.65 ± 0.08 μg/kg (600-fold lower than DDT-injected frogs), while dieldrin concentrations in control and DDT-injected frogs were 0.34 ± 0.07 μg/kg (145-fold lower than dieldrin-injected frogs). Concentrations of DDT in whole-body homogenates were an average of 2.3-fold lower at 20 weeks compared with the frog sampled on day 2, whereas dieldrin concentrations were lower by a factor of 3.4-fold. However, because only one individual

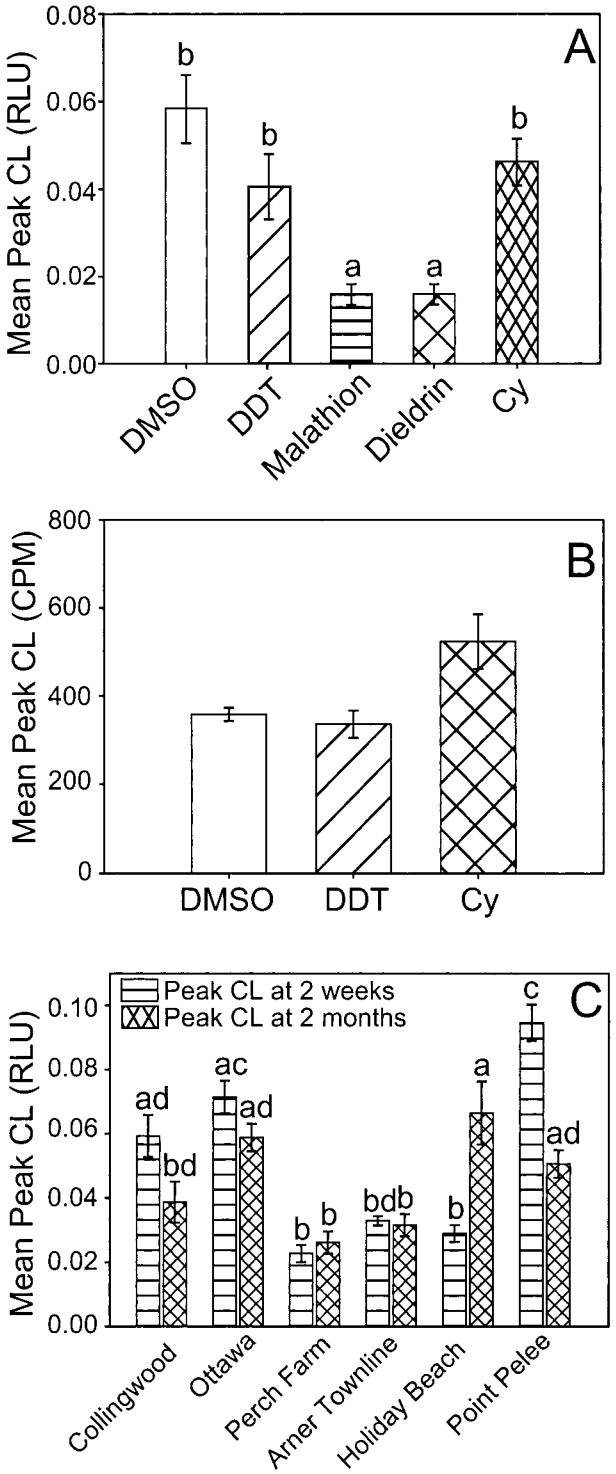

Fig. 4. (**A**) Zymozan induced-chemiluminescence in leopard frogs eight weeks after exposure to DMSO, DDT, dieldrin, malathion, and cyclophosphamide (Cy); $n = 5$ per group ($p \leq 0.0001$). (**B**) Zymozan-induced peak chemiluminescence assayed at eight weeks postcontaminant exposure in frogs immunized with keyhole limpet hemocyanin 5 d prior to contaminant exposure; $n = 5$ per group ($p = 0.05$). (**C**) Zymozan induced-chemiluminescence, assayed within the first month of capture, in field-collected leopard frogs from the regions near Ottawa ($n = 8$) and Collingwood ($n = 7$) and in Essex County, including the perch farm ($n = 7$), Arner Townline ($n = 11$), Holiday Beach ($n = 12$), and Pelee ($n = 12$) ($p \leq 0.0001$). All study sites are located in Canada. RLU = relative light units; columns with the same lowercase letter denote no statistical difference.

frog was sampled per treatment group at day 2, a quantitative estimate of elimination rates cannot be derived. Despite this, the data do suggest a high degree of persistence of both DDT and dieldrin in leopard frogs. Malathion and cyclophosphamide concentrations could not be determined because protocols for their measurement were not available. During the injection study, the weights increased in all frogs throughout the duration of the experiment. Over the 20 weeks of the experiment, the average weight gain for males was 16.0 g and 15.6 g for females. The mean lipid content of frogs at 8 weeks was 4.0 ± 1.5% and at 20 weeks was 4.3 ± 0.7%.

The results of the contaminant analysis for the field-study frogs are shown in Table 2. At the Point Pelee site, the low number of frogs collected only allowed two to be used for contaminant analysis. One of the frogs from Point Pelee (Point Pelee 1) exhibited elevated levels of DDT, 1,1-dichloro-2,2-bis(chlorophenyl) ethylene (DDD), and 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethane (DDE). These levels were close to those used in the injection study. The other frog from Point Pelee had moderately high values of DDT and DDE concentrations relative to the other sites. Dieldrin was also found to be high in these frogs and was of similar magnitude to the dieldrin concentrations used in the injection study. Holiday Beach had the next highest levels of DDE—three times the amount found in the Collingwood frogs. Dieldrin at this site was elevated slightly in two of three frogs. No other dramatic differences existed in the contaminant levels among the various groups. As expected, Ottawa and Collingwood groups showed the lowest pesticide burdens. The mean increase in the weights of the frogs during the two-month laboratory holding period was roughly similar for four of the sites (Ottawa, 2.8 ± 2.7 g; perch farm, 2.7 ± 2.5 g; Holiday Beach, 1.5 ± 2.1 g; and Point Pelee, 2.0 ± 2.8 g), with the Arner Townline and Collingwood frogs gaining more weight, 7.9 ± 8.6 g and 5.8 ± 3.3 g, respectively.

The results indicate that there are spatial differences in pesticide contamination of Ontario leopard frog populations and that pesticide residues in wild frogs approach the concentrations that suppressed immune function in the present study.

DISCUSSION

A functioning immune system is an absolute requirement for the survival of individuals. Populations containing many individuals with compromised immune systems will decline rapidly. Humoral immunity, a major arm of the vertebrate immune system, depends on the presence of antibodies to neutralize pathogens and to direct effector mechanisms that destroy pathogens. This investigation used injection of pesticides to demonstrate that exposure to a sublethal dose of certain pesticides, administered at levels that are present in wild frogs, altered aspects of the immune function in leopard frogs in a manner consistent with immunosuppressive drugs.

Effect of pesticides on amphibian humoral immunity

The injection study indicated that exposure to a single sublethal dose of DDT, malathion, or dieldrin dramatically reduced, in some cases virtually eliminated, the production of KLH-specific antibodies. A gradual recovery of the ability to produce specific antibodies was apparent over several weeks, but complete recovery did not occur until week 20 (Fig. 2A). This lack of antibody response would allow extracellular path-

Table 1. The concentration of the stock solution of the pesticides and cyclophosphamide dissolved in DMSO used for the injection study along with the expected and actual concentrations of DDT and dieldrin found in the frogs 2 d after exposure (n = 1) and 20 weeks after exposure (n = 5); ND = not determined; SD = standard deviation

Contaminant group	Stock solution (mg/L)	Expected concn. (ng/g)	Mean concn. 2 d (ng/g)	Mean concn. (±SD) 20 weeks (ng/g)	Mean concn. (±SD) non-injected (ng/g)
DDT	250	750	923	404.8 ± 36	0.65 ± 0.08
Dieldrin	25	75	49.6	14.4 ± 6.1	0.34 ± 0.07
Malathion	330	990	ND	ND	ND
Cyclophosphamide	3,000	9,000	ND	ND	ND

ogens infecting these frogs to proliferate to lethal levels within this time scale if unchecked by other mechanisms of immunity.

When the frogs received the antigen prior to the administration of pesticides, there was no effect on production of antibodies to the specific antigen (Fig. 2B). This indicates that the pesticides are not destroying immune capability, merely inhibiting it in the manner of immunosuppressive drugs. It also indicates that the pesticides do not inhibit the activation of B cells containing specific antibodies but instead inhibit the antigen presentation process that initially selects the specific B cell clones for expansion. We are currently investigating the effect of these pesticides on this process. These results suggest that wild frogs that have developed humoral immunity to pathogens would retain it following pesticide exposure but that new pathogens would pose a serious threat.

In the field study, different populations of frogs showed varying abilities to produce antibody in response to a specific antigen when assayed two weeks following capture. In agreement with the injection study, the Collingwood group, which was collected in an area that has not been actively exposed to pesticides, was capable of dramatically higher antibody responses than the other frog groups tested (Fig. 2B). When the same assay was repeated two months later, the antibody response of the groups had changed dramatically and all the groups except Point Pelee were capable of responding to the specific antigen to the same degree as the Collingwood group.

Table 2. Contaminant analysis of individual frogs collected from various areas of Ontario determined immediately after collection. ND = not determined; DDD = 1,1-dichloro-2,2-bis(chlorophenyl) ethylene; DDE = 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethane. All sites are in southern Canada and are shown on the map in Figure 1

Sample identification	*p,p′*-DDE (ng/g wet wt)	*p,p′*-DDD (ng/g wet wt)	*p,p′*-DDT (ng/g wet wt)	Dieldrin (ng/g wet wt)
Point Pelee 1	491.46	17.12	121.02	42.32
Point Pelee 2	16.89	0.20	2.94	56.58
Holiday Beach 1	5.39	ND	0.51	1.12
Holiday Beach 2	6.44	0.16	0.95	6.60
Holiday Beach 3	7.57	0.52	1.07	6.82
Arner Townline 1	2.37	ND	0.36	1.98
Arner Townline 2	2.66	ND	0.42	3.92
Arner Townline 3	3.76	0.29	0.78	4.72
Perch farm 1	1.45	0.12	0.27	0.36
Perch farm 2	1.58	ND	0.45	0.34
Perch farm 3	1.35	ND	ND	0.32
Perch farm 4	1.28	ND	ND	0.48
Collingwood 1	2.22	ND	0.15	0.56
Collingwood 2	2.92	ND	0.82	0.70
Collingwood 5	2.27	ND	ND	0.24
Ottawa 3	1.01	ND	ND	0.34
Ottawa 4	0.78	ND	ND	0.22
Ottawa 5	1.09	ND	ND	0.40

These results suggest that, when the frogs were first captured, the ability of the different populations to produce antibodies was impaired in all the groups when compared with the response of the Collingwood group. The recovery of humoral immunity when removed from pesticide exposure in this experiment is consistent with our observations in the injection study.

This assay has not been performed previously for amphibians and studies of this kind in mammals were performed using different detection technologies, so it is hard to correlate our results with those data. In addition, two groups performing this type of experiment on mice with DDT reported conflicting effects on specific antibody production [25,26]. We intend to perform a parallel study using this assay on frogs and mice, measuring the results in exactly the same fashion in order to determine the applicability of these observations to other species, particularly humans.

Effect of pesticides on amphibian cellular immunity

The DTH responses of the pesticide-exposed groups in the injection study appeared higher than those of the control group, although they were not statistically higher (Fig. 3A). It is possible that this could be a compensatory reaction to the loss of antibody production in these frogs. The results of contamination are not always the suppression of the immune response; some endpoints can be stimulated. For instance, autoimmunity and hypersensitivity are possible outcomes of stimulated immune system function [27]. It is known that cyclophosphamide enhances cellular immune responses when administered prior to the antigen [28], which was the case here. Once again, the pesticides are acting in a manner consistent with immunosuppressive drugs. The reverse-order administration study was undertaken to see if this similarity would continue if the antigen was administered prior to the pesticides and cyclophosphamide, which suppresses cellular responses when administered in this order [29]. In this experiment, the DDT- and cyclophosphamide-treated groups did show less reaction than the control group, although it was again not statistically significantly different (Fig. 3B). This demonstrates once again the similarity in action of pesticides and immunosuppressive drugs. The field study demonstrated that there are also significant differences in cellular immune capacity in different populations of Ontario frogs. The observation that Collingwood frogs showed the lowest DTH response while frogs from areas that contain more pesticides have a higher DTH response (Fig. 3C) is consistent with our injection-study observation that pesticide exposure prior to encountering an antigen enhances DTH responses (Fig. 3A).

In this study, although frequently not significant, the overwhelming trend showed that pesticides enhanced the DTH reaction in exposed frogs in comparison with the control group.

Strong dose–response relationships for organochlorines, including individual polychlorinated biphenyl congeners, and suppressed T-cell–mediated immunity have been reported in birds [30], seals [31], and DDT-exposed mice [32]. Other studies, however, have demonstrated increases in DTH skin reaction exposure to contaminants. For instance, one study examining the effect of DDT on the DTH reaction of rats to bovine serum albumin found that exposure elevated the response [33]. Perhaps the exact effect of pesticides on the DTH reaction—suppression or enhancement—is species specific.

Effect of pesticides on amphibian innate immunity

Our assay for innate immune function, the production of oxygen radicals by activated neutrophils from whole blood in response to a stimulus, yielded results similar, but not identical, to the assays for humoral and cellular immunity in all three experiments. Malathion and dieldrin suppressed neutrophil activation in the injection study (Fig. 4A). The DDT and cyclophosphamide groups did not have a significantly smaller response than the control group, but this assay was performed only once eight weeks after exposure, so we cannot tell if there was a suppression of neutrophil activation earlier that has recovered or if this is truly indicative of a lack of effect. In the reverse-order administration study, the DDT and cyclophosphamide did not inhibit neutrophil activation (Fig. 4B), although they had not significantly inhibited it in the injection study. We were unable to test dieldrin, which may have shown a reversal in effect similar to that seen for the other assays, due to the limited number of frogs available for this study. Finally, while the field study demonstrated that there are spatial differences in neutrophil activation capabilities of Ontario frogs, four of the six groups did not show a significant change in this capability after two months in captivity (Fig. 4C), an observation that contrasts the data from the injection study. In the two groups that did change in this capacity during the holding period, one increased and one decreased. This may indicate that, while innate immune response capabilities vary among populations, pesticides do not affect them to the same degree as the two arms of the adaptive immune system.

Studies on the effect of pesticides on human neutrophil function also provide conflicting results. Queiroz et al. [34] reported that organophosphate and carbamate pesticides decrease the ability of neutrophils to produce oxygen radicals when studying workers exposed to these compounds, but studies of neutrophil function in vitro show that neutrophil activation is enhanced by toxaphene [35] as well as lindane and dieldrin [36,37]. This may mean that the presence of other cell types in vivo, perhaps even cells of the adaptive immune system, modify the response of neutrophils to pesticides. On the other hand, it may just indicate that the response is variable and depends on several factors.

Correlations between pesticide burden and immune function

There was no direct correlation between the concentration of pesticides measured in each experimental group of frogs and their performance in immune function assays. In general, however, frogs with large pesticide burdens demonstrated changes in immune function consistent with the presence of immunosuppressive drugs.

Contaminant levels in the injection-study frogs dropped between the time of injection and week 20 (Table 1) but still persisted in tissues at concentrations well above those (145–640-fold higher) encountered in the ambient laboratory population. This contrasts with the immune function assays, which showed very little antibody production capability during the first eight weeks following injection (Fig. 2) but nearly full recovery of antibody production between weeks 8 and 20. We examined whole-body concentrations of these compounds, but it may be possible that changes in the internal distribution and recompartmentalization of the injected dose over the course of the study better correlated with the recovery of immune function. We are currently examining the elimination and compartmentalization of pesticides by leopard frogs in greater detail.

The field study also allowed us to examine the relationship between organochlorine contaminant burdens and immune function. As observed in the injection study, no direct correlations between organochlorine residues and immune function emerged from the data. However, spatial differences in both contaminant burden and immune capability were apparent and may allow some explanation of the results. The Point Pelee frogs had the highest burdens of DDT (plus DDT metabolites) and dieldrin (Table 2) and also showed a low antibody-production capability that failed to recover after two months in captivity—the only group that did so. Other studies have also demonstrated high organochlorine concentrations in frogs collected from Point Pelee and western Lake Erie. Gillan et al. [38] reported DDE concentrations in the range of 125 to 754 ng/g in frogs collected from the north shore of Lake Erie [38], while Russell reported in 1995 that a single frog from Point Pelee had 1,001 ng/gm DDE and 199.8 ng/gm dieldrin [9]. The Collingwood frogs showed the least evidence of immunosuppressive effects but did not differ significantly in the amounts of measured pesticides present in frogs from the perch farm and Ottawa frogs. These latter groups also showed some immunosuppressive effects. The Holiday Beach and Arner Townline animals exhibited immunosuppression, but they did have higher levels of DDE and/or dieldrin than Collingwood frogs. The differences in immune function may also be related to compounds we did not or could not measure.

All of the above data show that, while pesticides have detrimental effects on immune function, the simple presence or absence of these compounds or a given concentration of these compounds in an animal is not indicative of immune effects. We are currently examining the effect of different routes of exposure to these compounds on immune function, particularly exposure through food sources and dermal contact. This study is the first to show that immunological status of adult frogs can be measured in a nonsacrificial manner and that spatial differences in immune function are present in frog populations. It has also demonstrated that, for leopard frogs, pesticides do act as immunosuppressive agents at sublethal doses—doses that are present in wild frogs. The immunosuppressive effects of pesticides may be contributing to amphibian declines by rendering exposed populations susceptible to common pathogenic organisms.

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