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Effects of Inhalation Exposure to a Binary Mixture of Benzene and Toluene on Vitamin A Status and Humoral and Cell-Mediated Immunity in Wild and Captive American Kestrels

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Benzene and toluene are representative volatile organic compounds (VOC) released during production, storage, and transportation associated with the oil and gas industry and are chemicals of concern, as they are released in greater and possibly more biologically significant concentrations than other compounds. Most studies of air pollution in high oil and gas activity areas have neglected to consider risks to birds, including top-level predators. Birds can be used as highly sensitive monitors of air quality and since the avian respiratory tract is physiologically different from a rodent respiratory tract, effects of gases cannot be safely extrapolated from rodent studies. Wild and captive male American kestrels were exposed for approximately 1 h daily for 28 d to high (rodent lowest-observed-adverse-effect level [LOAEL] of 10 ppm and 80 ppm, respectively) or environmentally relevant (0.1 ppm and 0.8 ppm, respectively) levels of benzene and toluene. Altered immune responses characteristic of those seen in mammalian exposures were evident in kestrels. A decreased cell-mediated immunity, measured by delayed-type hypersensitivity testing, was evident in all exposed birds. There was no effect on humoral immunity. Plasma retinol levels as measured by high-performance liquid chromatography (HPLC) analysis were decreased in wild and captive kestrels exposed to the rodent LOAEL for combined benzene and toluene. This study indicates that American kestrels are sensitive to combined benzene and toluene. The study also illustrates the need for reference concentrations for airborne pollutants to be calculated, including sensitive endpoints specific to birds. Based on these findings, future studies need to include immune endpoints to determine the possible increased susceptibility of birds to inhaled toxicants.

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A fully functioning and protective immune system is dependent upon constant and effective communication between the humoral (B lymphocytes), cell-mediated (T lymphocytes), and innate components. This is achieved by various cytokines, hormones such as thyroxine (T4) and triiodothyronine (T3), and vitamin A derivatives. Thyroid hormones (THs) are responsible for growth, differentiation, and metabolism, as well as overall hormonal balance within an individual (Rolland, 2000), while vitamin A plays a role in antibody production, T-lymphocyte responses, phagocyte reactions, and cellular maturation and differentiation (Friedman and Sklan, 1997). When the immune system is functioning properly, an individual who encounters a bacterial, viral, or other foreign antigenic stimulus will elicit a strong antibody and/or cell-mediated response with the intent of neutralizing and eliminating that particular antigen. Plasma concentrations of thyroxine and retinol are linked since they share a common carrier protein, transthyretin (TTR) (Brouwer et al., 1990; Rolland, 2000).

Immunotoxicology is the study of adverse effects on the immune system from exposure to various environmental chemicals, including drugs, biological materials, and environmental pollutants (Luster & Blank, 1987). Many classes of pollutants are immunotoxic in wildlife (Fairbrother et al., 2004; Fox et al., 1998). However, studies of immune effects of airborne contaminants appear to be limited to mammals (Rozen et al., 1984; Snyder, 2004) with few exceptions (Carmalt, 2005).

Chemically, volatile organic compounds (VOC) associated with flare gas emissions are unsaturated cyclic (aromatic) hydrocarbons that contain one or more benzene rings (ATSDR,

2000). Benzene, toluene, ethylbenzene, and xylene (BTEX) are used as representatives of this group. Benzene and toluene in particular are hazardous due to their inherent toxicity in mammals, while their wide use in industry and high volume of production lead to substantial environmental releases (Robinson et al., 1997). Benzene is a well-known immunotoxicant targeting maturing B- and T-lymphocyte populations in the bone marrow (Rozen et al., 1984; ATSDR, 2006; Snyder, 2000). The lowest-observable-adverse-effect level (LOAEL) for these immune effects in mice is 10 ppm (Rozen et al., 1984). When animals are coexposed to toluene and benzene, the adverse immune effects are diminished. Toluene is thought to be protective against benzene-related immunotoxicity by preferentially binding CYP2E1, the biotransformation enzyme responsible for benzene metabolism, thereby decreasing the toxic metabolite level from benzene metabolism in the body (Purcell et al., 1990).

Environmental risk assessments are broadening to include evaluations of avian species exposed to gaseous and particulate materials (Mineau, 2002; Carmalt, 2005; Irvine, 2004). Since the avian respiratory tract is fundamentally different from that of rodents, the effects of gases on birds cannot validly be extrapolated from rodent studies (Briant & Driver, 1992; Brown et al., 1997). Anatomical and physiological differences in the avian respiratory tract include unidirectional airflow, separation of tidal expansion and gas exchange, thinner-gas-exchange tissues, and lack of resident alveolar macrophages in gas-exchange areas, making it more sensitive to inhaled substances than the mammalian lung. Respiratory-tract differences result in approximately double the absorption of air contaminants by the avian lung compared to the mammalian lung (Brown et al., 1997). Over the past decades birds, galliforms and waterfowl, were used as models for inhalation studies but only in the context of effective routes of vaccine administration (Myers & Arp, 1987; Eidson & Kleven, 1976). A limited number of inhalation studies focused on birds as target animals. European starlings were used in field studies to determine the effects of emissions from flare stacks associated with the oil and gas production industry (Carmalt, 2005; Irvine, 2004), and goldfinches were studied regarding toxicity associated with coal-fired power plant emissions (Gorriz et al., 1994).

Considering the high concentration and immunotoxic potential of benzene and other halogenated hydrocarbons such as toluene in ambient air on the prairie and boreal plain regions of Saskatchewan and Alberta (Waldner et al., 2001), plus the prevalence of raptors in these areas (Hoffman & Smith, 2003; Kirk & Hyslop, 1998), a laboratory-based study was designed to test a bird species. The objectives were to (1) address the lack of avian-specific LOAEL in determining reference concentrations for airborne pollutants, (2) determine the effects of benzene and toluene on B-cell- (antibody production) and T-cell-mediated (delayed-type hypersensitivity) immunity, and (3) look for alterations in thyroid hormone and vitamin A levels in American kestrels (*Falco sparverius*). The American kestrel serves as a valid and useful sentinel owing to its small size, its nonendangered status, its relatively easy maintenance in captivity, its top trophic position (Wiemeyer & Lincer, 1987), and its value as a model for other larger prairie-inhabiting raptors. The study assessed the variables stated already, after exposures to binary mixtures of environmentally relevant levels of benzene (0.1 ppm) and toluene (0.8 ppm), as well as to rodent LOAEL levels of benzene (10 ppm) and toluene (80 ppm) administered via inhalation.

MATERIALS AND METHODS

Exposure of American Kestrels to Benzene and Toluene

Wild Birds (Year 1)

Because of no availability of captive kestrels in May 2005, 23 male American kestrels of varying ages were captured using Bal-chatri traps from a 100-km radius of Prince Albert, Saskatchewan (SK), Canada. The birds were transported to a flight barn 7 km from Saskatoon, SK, where they were acclimated for 5 wk before being transported to the Animal Care Unit (ACU) at the University of Saskatchewan (U of S) (Saskatoon, SK). Kestrels were subsequently divided by weight into two dose groups: control group (n = 11) and high dose group (n = 12). Treatment groups were housed separately in windowless rooms with shavings covering the floor, two equal-height rope perches, a water bath, greenery in the form of tree branches, and three feeding stations. Each room was on a 12-h light/dark cycle and feeding times were staggered at 10:30 (control group) and 12:30 (high group). The daily diet consisted of 1½ frozen then thawed day-old cockerel chicks per kestrel (Anstey Hatchery, Saskatoon, SK). Grouped birds were acclimated to the new setting for 2 wk prior to commencing exposures. Baseline mass and body condition were recorded 1 wk prior to dosing. Over the next 6 wk of challenge exposures, birds were weighed weekly and bled after wk 1, 2, 4, 5, and 6 (necropsy).

Captive Birds (Year 2)

In May 2006, 32 male American kestrels, 2 to 3 yr of age, some of which had been previously exposed in ovo to polybrominated diphenyl ethers (PBDE), were available for our study from the Avian Science and Conservation Centre, McGill University (Ste Anne de Bellevue, QC). All birds were divided among the benzene/toluene treatment groups: control (n = 11), low (n = 10), and high dose (n = 11). Birds previously exposed to PBDE were evenly distributed among the different benzene/toluene dose groups. Groups were placed in separate flight rooms for the duration of the study. Each room had screened windows, a feeding bench, water bath, two rope perches, and shavings covering the floor. Kestrels were fed 1½ frozen then thawed day-old cockerel chicks per day.

Birds were weighed weekly and blood samples were collected before exposure and at wk 1, 4, 5 and 6 (necropsy) of

exposure. Approximately 1 ml of blood (<1% body weight) was collected from the jugular vein using 7.5% ethylenediamine tetraacetic acid (EDTA) (2005) (Sigma, Aldrich St. Louis, MO) or heparin sodium (2006) (Hepalean, Organon Teknika, Toronto, ON) coated syringes and 28-gauge needles. Samples were placed on ice in 1.5-ml Eppendorf vials. Blood was centrifuged at 800 × g for 5 min. Plasma was removed and frozen at –40°C for future analysis. In accordance with Canadian Council on Animal Care guidelines, the experimental protocols were approved under University of Saskatchewan protocol number 20050025, and University of McGill protocol number 5236.

Benzene and Toluene Concentrations

High exposure concentrations were chosen based on the current rodent LOAEL data for both benzene (10 ppm) and toluene (80 ppm) (ATSDR, 2000, 2006). Low exposure concentrations (yr 2 only) were based, in part, on the average concentrations of benzene (0.085 ppm) and toluene (0.085 ppm) in spring and summer months near flare stacks in southern Alberta (Carmalt, 2005), but to maintain the ratio of benzene:toluene (1:8) as in the high dose, final low-dose concentrations were benzene 0.1 ppm and toluene 0.8 ppm. Praxair Distribution produced benzene/toluene gas mixtures (Geismer, Los Angeles, CA). High-pressure aluminum cylinders with a 4.12-m^3 capacity were filled with a breathing-grade air carrier gas and 10 ppm benzene, 80 ppm toluene (Al BZ10MT1C-AS) (Years 1 and 2), or 0.1 ppm benzene and 0.8 ppm toluene (Al BZ0.1MT1C-AS) (Year 2). Mixtures were verified to ±5% using gas chromatography with flame ionization detection (GC-FID). Ultra-high-pressure steel cylinders, volume of 6.43 m^3, containing breathing grade air (AI BR-K), were also purchased from Praxair (Saskatoon, SK [2005], St Laurent, QC [2006], Canada) for control group exposures.

Daily Standard Operating Procedure and Dose Time

The same standard operating procedure was used for both wild and captive kestrels with the exception of adding the low-dose group in yr 2. Between 0800 and 0830, Monday through Friday, control group birds were captured and transported to the inhalation chamber (Olsgard & Smits, 2007), individuals were randomly placed, tail first, into the holding chambers, and each lid was firmly secured. Once birds were in place, the breathing-grade air cylinder, vacuum pump, inlet gas line, ball valve, and gas regulator were all turned on and the flow was adjusted to 5 L/min (lpm). After the dosing session plus equilibration time was complete, all gas lines were turned off and the birds were returned to their holding rooms with fresh water baths and food. In yr 1, the high dose group followed the control group, and daily exposure was 1 h plus chamber equilibration time, for a total of 34 h in the inhalation chamber over 28 d. In yr 2, the control group (breathing grade air) was followed by the low-dose group, which was followed by the high-dose group, with 1½ h exposure per day for 27 days, totaling 40.5 h, including equilibration time. The total dosing time was increased in yr 2, once it was confirmed that there was no overt toxicity or undue distress experienced by the birds in yr 1.

Humoral Immune Response

In yr 1and 2, birds were immunized after 4 wk of exposure, and received booster immunizations 7 d later, with blood samples being collected just prior to each immunization and 7 d after the booster. In yr 2, all the birds had been vaccinated with dinitrophenol–keyhole limpet hemocyanin (DNP-KLH), (Calbiochem, Terochem Laboratories Ltd, Edmonton, AB) as juveniles a year earlier in previous studies of in ovo exposure to PBDEs. Therefore, the first vaccination during our study in 2006 would have elicited a secondary (immunoglobulin G, IgG) antibody response; thus, blood was collected for antibody determination 7 d after the immunization. Sensitization was accomplished using a DNP-KLH vaccine formulated per bird as follows: 6.7 µl stock DNP-KLH in 3-[*N*-morpholino] propane sulfonic acid (MOPS) (7.5 mg/ml) was added to 30.8 µl sterile phosphate-buffered saline (PBS) with 37.5 µl of Emulsigen-Plus adjuvant (MVP Laboratories, Ralston, NB). The entire solution was emulsified on ice using an 18-gauge needle attached to a syringe under a laminar flow hood.

Details of the immunization were as follows: A baseline blood sample (yr 1) was taken prior to two subcutaneous (50 µl each, between shoulder blades, inner thigh region) and one intramuscular injection (25 µl in breast muscle) of the DNP-KLH vaccine per bird. Seven days after the initial vaccination a blood sample was drawn, and a booster injection was administered as previously described. The final blood samples were taken via intracardiac puncture while the bird was anesthetized with halothane prior to euthanasia. In yr 2, the blood sample was taken 7 d after immunization to determine the secondary antibody response.

The anti-DNP-KLH antibody response was determined using an enzyme-linked immunosorbent assay (ELISA). A total of 100 µl of 0.5 µg/ml DNP-KLH in carbonate buffer (0.05 *M*, pH 9.5) was added to all wells of a 96-well Nunc-Immuno Maxisorp microtiter plate (Canadian Life Technologies, Inc., Burlington, ON) and incubated at 4°C for 15–16 h. After rinsing the plate 4 times with 0.05 *M* PBST (pH 7.2: 0.05% Tween 20) and tapping dry, residual binding sites were blocked using 100 µl bovine serum albumin (BSA) (0.25% BSA in 0.05 *M* PBS, pH 7.2: 0.05% Tween 20 [PBS-T]), incubated for 30 min at 38°C, and washed as described. Next, 100 µl of a 1:400 dilution of laboratory-produced rabbit anti-kestrel antibody (Smits & Baos, 2005) in PBS-T was added to the plate, incubated for 30 min at 38°C, washing was repeated, and 100 µl of a 1:800 dilution of goat anti-rabbit peroxidase-conjugated secondary antibody (Sigma Aldrich, St. Louis, MO) in PBS-T was added to all wells,

incubated for 30 min at 38°C, and washed. Finally, 100 µl of 2,2'-azino-bis(3-ethylbanzo-thiazoline-6-sulfonic acid) (ABTS) (Mandel Scientific, Guelph, ON) was added to all wells and incubated in the dark for 5 min. Using 1% sodium dodecyl sulfate (SDS) in ultrapure water, the reaction was stopped and plates were read on a BioRad 3550 microplate reader with a 405-nm filter (BioRad Laboratories, Mississauga, ON, Canada) to determine the optical density (OD_{405}) of each sample. Data were analyzed using microplate manager version 4.0 (BioRad Laboratories, Mississauga, ON). Prior to running plasma samples from all test birds, pooled plasma from five kestrels, representing all exposure groups, provided a "standard" plasma sample. This standard was used as a control on every plate, against which all other samples were compared. Individual kestrel antibody levels were then expressed as percent of standard run on that plate (Figures 1 and 2).

Cell-Mediated Immunity Using the Delayed-Type Hypersensitivity Test

To elicit a proper delayed-type hypersensitivity (DTH) response, a measure of integrated cell-mediated immunity, the animals need prior sensitization to the test compound. Prior exposure to DNP-KLH for the antibody response provided this sensitization and a DTH response was tested a minimum of 2 wk postvaccination. One wk prior to DTH challenge each bird had a 1-cm patch on the mid-patagium (wing web) of the left wing cleared of all feathers and down. On the last day of benzene and toluene exposures, birds were weighed and the patagium thickness was measured (mean of three measurements, mm) using a micrometer (Dyer OD gage 0·01 mm, The Dyer Company, Lancaster, PA), swabbed with alcohol, and injected with keyhole limpet hemocyanin (mcKLH) at 20 µg in 50 µl PBS using a 30-gauge needle. Injection sites were marked with a waterproof marker. Twenty-four hours later, birds were weighed and the same investigator measured injection sites. The DTH response was the difference between the post and pre skin thickness.

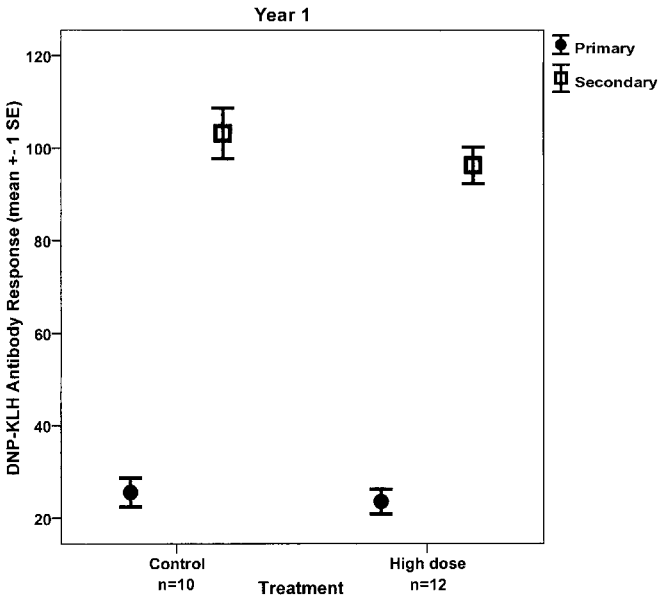

FIG. 1. Year 1: Primary and secondary antibody responses to DNP-KLH (mean ± SE) in wild American kestrels exposed to breathing-grade air (control) or high-dose (benzene 10 ppm, toluene 80 ppm) contaminated air.

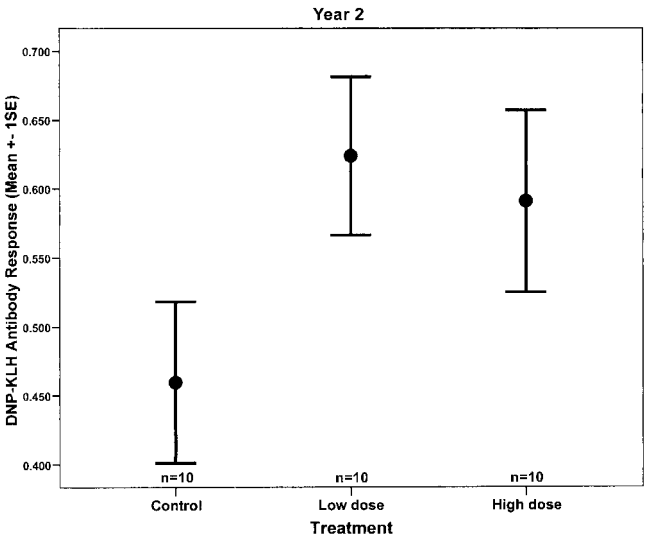

FIG. 2. Year 2: Secondary antibody responses to DNP-KLH (mean ± SE) in previously immunized captive American kestrels exposed to breathing-grade air (control), or low-dose (benzene 0.1 ppm, toluene 0.8 ppm) or high-dose (benzene 10 ppm and toluene 80 ppm) contaminated air.

Vitamin A Levels

Plasma Retinol

Plasma samples collected from wild and captive birds before and after dosing with benzene and toluene were stored at –20°C in amber-colored, Eppendorf microcentrifuge tubes until analysis. Due to the photo-oxidation properties of retinoids, all samples were analyzed in an enclosed, dark workspace with a 20-W gold fluorescent light bulb (Bulbtronics, Inc., Farmingdale, NY). All solvents, unless otherwise stated, were high-performance liquid chromatography (HPLC) grade (99%) and purchased from VWR International or EMD Chemicals. All samples were extracted and analyzed as follows. Plasma was thawed in the dark and microcentrifuged for 5 min × 2300 RCF to remove all fibrous debris. Seventy-five microliters of plasma was added to a 10-ml screw-top Oak Ridge Teflon centrifuge tube (VWR International, Mississauga, ON). To the plasma, 50 µl of an 8-µg/ml retinol acetate (RA) internal standard (R4632 Sigma Aldrich, Oakville, ON) was added for a final RA concentration of 320 µg/L per sample, which was then shaken by hand. Next, 250 µl of 100% ethanol containing 15 mg/ml 2,6-di-*tert*-butyl-4-methylphenol (BHT) (Sigma Aldrich, Oakville, ON), was added and vortexed for 20 s.

Lastly, 1.5 ml hexane was added, and the mixture was vortexed for 2 min and centrifuged at 4°C, 1258 RCF, for 2 min until the organic phase had separated. After centrifugation the upper hexane phase with the extracted retinoids was aspirated with glass pipettes and transferred to an aluminum-foil-covered 5-ml glass test tube. The step just described was repeated twice. Next, hexane layers were removed and evaporated under nitrogen gas for approximately 30 min (Praxair, Saskatoon, SK) and the residual pellet was then dissolved in 500 µl methanol and ultrasonicated for 5 min in a water bath. This sample containing all lipid-soluble vitamins was collected into a 1-ml syringe and filtered using a 0.2-µm PTFE acrodisc premium syringe filter (PALL Life Sciences, VWR International, Mississauga, ON) into a 1.8-ml amber-colored, screw-thread glass HPLC vial with 9-mm step vial closures. The extracted retinoids were then analyzed by HPLC (PDA model 330, Fluorometer model 363, autosampler model 410, Varian Inc, Mississauga, ON) as follows: Chromatography apparatus consisted of a C18 column (Microsorb-MV, 150 mm × 4.6 mm ID, Varian, Mississauga, ON) with a 100% methanol mobile phase at a flow rate of 1 ml/min. Eluants were monitored by ultraviolet absorption using a diode array wavelength detection and fluorescence detection. Fifty microliters of sample was injected by an autosampler with an automatic postinjection wash phase (60 methanol:40 water) after each sample, and reached the column via a trivalve pump (model 230, Varian, Inc., Mississauga, ON) with a dynamic mixing stage. The wavelength for ultraviolet (UV) absorption was 325 and total run times were 7 min with a peak retention time for retinol at ~3 min and retinyl acetate at ~4 min per sample. Daily calibration curves were prepared using a series of dilutions of retinol (Sigma Aldrich, Oakville, ON) (Table 3.1) dissolved in mobile phase with the same concentration of internal standard (retinol acetate 800 µg/L) as samples. Data were generated using Galaxy software version 1.8. Actual concentrations of retinol (µg/L) were calculated using linear regression of curve data using measured retinol to internal standard peak area ratios. Only calibration curves with a correlation coefficient (R^2) greater than .95 were accepted.

Liver Retinyl Palmitate

Liver samples were frozen in liquid nitrogen and stored in a –80°C freezer in clear screw-cap cryovials until extraction and analysis based on a revised protocol (Milne & Botnen, 1986). To prevent photo-oxidation of retinoids, all samples were handled under with yellow spectrum light. Liver samples were thawed and weighed before extraction. Approximately 0.7 mg liver was ground in a large mortar and divided into 2 equal portions (0.2 mg homogenized liver) that would serve as duplicates. Samples were then ground with anhydrous sodium sulfate (FW 142.02 g/mol) in a small mortar until it became a homogeneous dry pink powder. The liver sodium sulfate mixture was then transferred to a Teflon tube (VWR International, Mississauga, ON) and 800 µl of a 10-µg/ml retinyl acetate internal standard (320 µg/L final concentration) (R4632 Sigma Aldrich, Oakville, ON) was added. Next, 7 ml of a 9:1 methanol:dichloromethane (MeOH:DCM) mixed organic extraction solvent was added, and the solution was vortexed and ultrasonicated in a water bath. The mixture was then centrifuged at 1258 RCF at 4°C for 2 min and the organic phase was poured into an aluminum-covered volumetric flask. The extraction phase was repeated 2 more times, each involving the addition of 7 ml of MeOH DCM, centrifugation, and removal of the organic phase. After all the organic layers containing the retinyl palmitate had been collected, a volumetric flask was filled to 25 ml final volume with MeOH:DCM. The sample was drawn into a 1-ml syringe, filtered with a 0.2-µm PTFE acrodisc premium syringe filter (PALL Life Sciences, VWR International, Mississauga, ON), and expelled into a 1.8-ml amber-colored screw-thread glass HPLC vial with 9-mm step vial closures. The vial containing the extracted retinyl palmitate was then analyzed by HPLC as described earlier, with the following differences: The chromatography apparatus consisted of a C18 column (described earlier) with a 100% methanol to 30% dichloromethane:70% methanol gradient mobile phase at a flow rate of 1–1.5 ml/min. The wavelength for ultraviolet (UV) absorption was 325 nm and the total run time was 13 min with a peak retention time for retinol acetate at ~4 min and retinyl palmitate at ~10 min. A calibration curve was prepared daily using a series of dilutions of retinyl palmitate (Table 3.2) dissolved in mobile phase with the same concentration of internal standard (retinol acetate 320 µg/L) as samples. Data were generated using Galaxy software version 1.8 and the same protocol as already described. Actual concentrations of retinol palmitate are reported on a wet weight basis (µg/L).

Statistical Analysis

Data from exposed and control groups were analyzed using SPSS (version 15.0, SPSS, Inc., Chicago). Data were tested for normal distribution using nonparametric Kolmogorov–Shmirnov analysis and homoscedasticity (homogeneity of variance) using Levene's test. Any p value < .05 was log-transformed and reanalyzed using a one-way analysis of variance (ANOVA). If transformation was not possible the data were analyzed using a Kruskal–Wallis and/or chi-squared nonparametric test with a Mann–Whitney model to compare groups. For normally distributed data a descriptive summary was generated and outliers (greater than mean ±2 SD) were eliminated (Boxenbaum et al., 1974). A one-way ANOVA was initially run on all data sets to determine any group effects, followed by a general linear model (GLM) with a univariate analysis (ANCOVA). Before ANCOVA analysis a GLM tested covariates for significant interactions. Nonsignificant covariates were iteratively removed until only significant covariates remained. Bonferroni or Fisher's least significant difference (LSD) post hoc analysis adjusted for experiment-wise error was used to

ensure the Type I error rate associated with multiple outcomes was $\alpha \leq .05$. Significance differences between control and dose groups were asserted at $p < .05$.

In yr 2 only, in addition to the benzene and toluene effects, the previous, indirect exposure to PBDE through dosing of their parents had to be considered in analyses. A two-way ANOVA was used to test for interactions. Any significant interaction excluded that variable from further analyses.

RESULTS

There were no significant differences between benzene/toluene-dosed and control kestrels in primary or secondary antibody responses in yr 1 (Figure 1), or secondary antibody responses in yr 2 (Figure 2). Delayed type hypersensitivity (DTH) responses (mm) to KLH were significantly suppressed in benzene/toluene-dosed wild kestrels in yr 1 (Figure 3) and captive kestrels in yr 2 (Figure 4). In yr 2, the high-dose group was significantly lower than controls and lower than the low-dose group, both of which were suppressed compared to control birds. The paired sample analyses of plasma retinol concentrations taken before and after benzene/toluene exposures are presented in Table 1. Plasma retinol levels after high-dose exposures were lower than predosing levels in both years. In yr 1, retinol levels decreased by 49.419 μg/L in high-dose birds, which was significantly more reduced than in control birds (13.271 μg/L). There were similar findings in yr 2, with the decrease in plasma retinol being significantly larger in high-dose birds (37.954 μg/L) compared with controls (–0.187 μg/L). There were no differences in hepatic retinyl palmitate levels with respect to dose group in either year (data not shown).

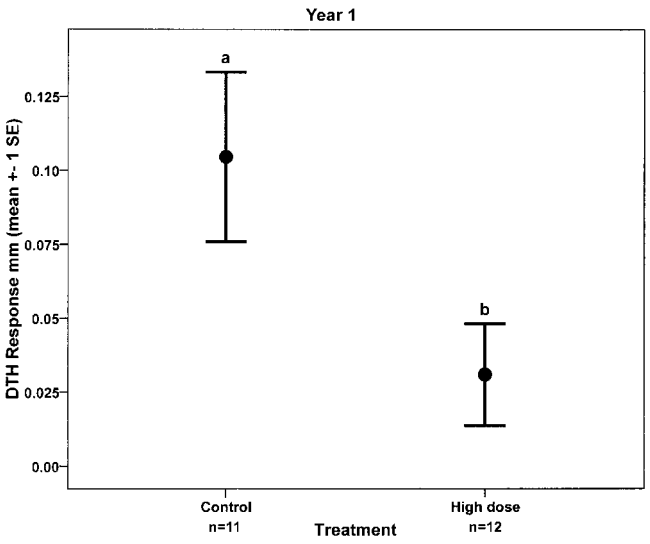

FIG. 3. Year 1: Delayed-type hypersensitivity (DTH) response (mm) in American kestrels exposed to breathing-grade air (control) or high-dose (benzene 10 ppm, toluene 80 ppm) contaminated air. Different superscripts indicate significant differences between groups.

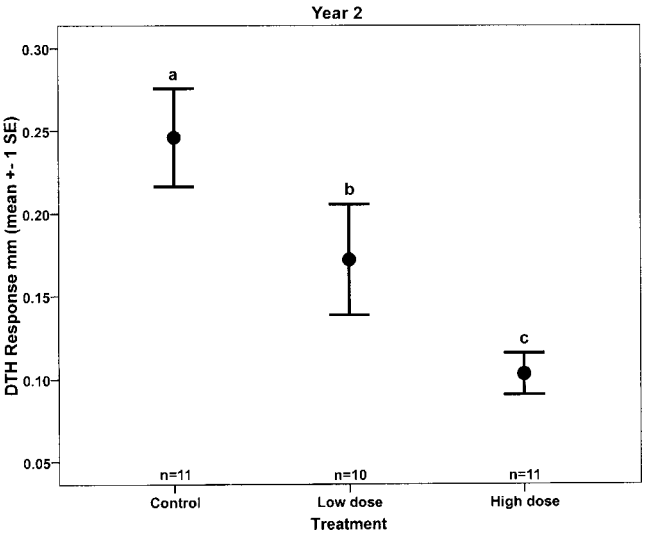

FIG. 4. The delayed-type hypersensitivity response (mm) in American kestrels exposed to breathing-grade air (control) or to low-dose (benzene 0.1 ppm, toluene 0.8 ppm) or high-dose (benzene 10 ppm and toluene 80 ppm) contaminated air in 2006. Different superscripts indicate significant differences among all groups ($p = .001$).

DISCUSSION

There was no effect of benzene and toluene exposure on B-cell-mediated immunity in either year. The cell-mediated immune response measured using the DTH response is a considerably more sensitive reflection of immune function (Tizard, 1992) than antibody response, since it measures the interaction among several arms of the immune system. The DTH test depends upon memory T lymphocytes responding to KLH antigenic stimulation, interacting with antigen-presenting macrophages and dendritic cells, all of which enhance the subsequent immunological reaction. The immunosuppression seen in these birds concurs with published findings in laboratory mammals, where CD4+ T helper lymphocytes are shown to be more sensitive to the adverse effects of benzene than CD8+ T suppressor lymphocytes (Robinson et al., 1997). There are few published reports in the avian literature examining the effect of contaminants on the DTH response. In waterfowl orally administered selenium compounds, the DTH response was suppressed (Fairbrother And Fowles, 1990). Numerous reports in mammals provide evidence that benzene suppresses cell-mediated immunity (Farris et al., 1997; Qu et al., 2002; Ross, 2000; Sul et al., 2002) and thus, the responses in our birds are consistent with those in mammals.

When one arm of the immune system is suppressed, another may overcompensate and produce an exaggerated response, or appear unaffected. A study by Smits et al. (1996) described the same immunomodulatory phenomenon in mink exposed to pulp mill effluent. They proposed that exposure to an immunotoxicant may enhance one arm of the immune response, while another is suppressed, a theory that was previously described

TABLE 1
Difference Between Predosing and Postdosing Plasma Retinol Levels (μg/L) in Wild (2005) and Captive (2006) American Kestrels Exposed 5 d/wk for 6 wk to Varying Concentrations of Benzene- and Toluene-Contaminated Air

Exposure	Decrease in plasma retinol (μg/L)	SD	Lower 95% CI	Higher 95% CI	*t*	df	Significance[a]
2005							
Control	13.271	46.741	−18.130	44.672	0.942	10	.369
High[b]	49.419	49.938	15.870	82.968	3.282	10	.008**
2006							
Control	−0.187	66.975	−83.347	82.973	0.006	4	.995
Low[c]	3.853	76.629	−60.210	67.916	0.142	7	.891
High[b]	37.954	44.939	0.384	75.523	2.389	7	.048*

[a]Two-tailed significance.
[b]10 ppm benzene, 80 ppm toluene.
[c]0.1 ppm benzene, 0.8 ppm toluene.

(Bretscher, 1981). It appears that exposure to benzene and toluene markedly affects T cell function, as was the case in this study. Lymphocytes generally are sensitive to the adverse effects of benzene's phenolic metabolites and have been used as one of the more sensitive indices with which to assess benzene-associated toxicity (Farris et al., 1997; Robinson et al., 1997). By provoking in vivo antibody production and testing the delayed-type hypersensitivity, the current study used functional tests of immunocompetence rather than only structural components of the immune system to understand the effects of benzene on the acquired immune systems of kestrels. Functional tests provide more insight into the potential capacity of these birds to deal with foreign antigens and possible infections.

Associations between contaminant exposures and suppressed immune function leading to increased disease, death rates, or decreased reproductive success have been described in many wildlife species. As examples, Glaucous gulls (*Larus hyperboreus*) exposed to organochlorines (OC) had a higher incidence of intestinal nematode infections (Sagerup et al., 2000), elevated polychlorinated biphenyl (PCB) concentrations were positively correlated with increased death rates of striped dolphins (*Stenella coeruleoalba*) in the Mediterranean sea during a morbillivirus outbreak (Aguilar & Borrell, 1994; Troisi et al., 2001), and altered immune functions in Caspian terns (*Hydroprogne caspia*) exposed to OC led to deformities, embryonic lethality, and decreased recruitment into the breeding population (Grasman et al., 1996; Mora et al., 1993).

Linking altered immune function with morbidity or mortality in wildlife based on laboratory-generated data needs to be done with care. Smits and Bortolotti (2001) found decreased phytohemagglutinin (PHA)-induced T-lymphocyte immunity and increased antibody responses in captive American kestrels exposed to PCB, findings that were supported by a field study that showed the same immunomodulation, a suppressed PHA response, and enhanced SRBC antibody response in Caspian terns (Grasman & Fox, 2001). Our study, using both wild and captive American kestrels in a laboratory setting, showed that both low and high benzene and toluene exposure resulted in suppressed T-lymphocyte activity no appreciable effect on B-lymphocyte immune responses. T-cell compromise might interfere with the ability of a wild bird population to deal with epizootics or other stressful events that occur in nature.

In both years of the current study there was a notable decrease in the circulating levels of retinol but no measurable effect on its stored form, hepatic retinyl palmitate. Benzene metabolism and Ah receptor association may explain this alteration. Once benzene enters the body it is metabolized by hepatic and pulmonary phase I enzymes and then transported to the bone marrow. In the marrow it is biotransformed by myeloperoxidase to semiquinone radicals, which stimulate the production of reactive oxygen species (ROS) (Gut et al., 1996). To deal with the increased free radical load, plasma retinol and vitamin A precursors may be redirected to the bone marrow, where they would be consumed by quenching ROS activity, thereby limiting their destructive effects (Sies & Stahl, 1995). Oxidative stress was not tested in these birds, so this possible explanation remains speculative. Cockerels fed to kestrels are a source rich in vitamin A (Rolland, 2000). It is very likely that the moderate level of toxic challenge to these kestrels did not deplete hepatic vitamin A stores, in part due to compensation by a high vitamin A diet.

Reduced plasma retinoids in high-exposure birds may also play a role in suppression of the cell-mediated response. Low vitamin A results in reduced antibody production and defective T-lymphocyte responses (Friedman & Sklan, 1997). The cell-mediated immunity (CMI) was significantly lower in birds with decreased retinol levels, an important finding considering that the immune function is dependent upon adequate levels of retinol. Reductions in vitamin A analogues were also reported in birds exposed to dioxins and furans (Spear et al., 1992),

PCB (Grasman et al., 1996), various aryl hydrocarbon (Ah)-inducing chemicals (Bishop et al., 1999), and PBDE (Fernie et al., 2005b). Mechanistically, this appears to be a displacement of retinol from transthyretin, in conjunction with increased phase II metabolism, resulting in accelerated clearance of retinol, decreased resorption by the kidney, and increased mobilization of hepatic stores, all resulting in decreased retinyl palmitate levels in the liver (Fernie et al., 2005b).

CONCLUSION

In the American kestrels studied here, alterations of immune function after exposure to benzene were similar to that described in mammals. However, the effects of toluene in this binary mixture, whether additive, synergistic, or competitive, are unknown. Although humoral immunity remained unaffected, cell-mediated immune function was clearly a target for benzene toxicity, with the most pronounced effects noted in high-dose individuals. Kestrels exposed to the rodent LOAEL possessed the greatest degree of cell-mediated immunosuppression, with corresponding decreases in plasma retinol levels. Considering the differential and decreased sensitivity of B cells, together with increased impacts on T-cell function and vitamin A metabolism, the biological consequences of inhalation exposure to volatile organic compounds in birds warrant further evaluation. The American kestrel has proven to be a sensitive model for benzene toxicity. The adverse effects seen here confirm the sensitivity of birds to inhaled toxicants, likely related to their unique respiratory physiology. If the immunomodulation witnessed in this study is occurring in wild birds, compromised resistance to infectious challenges might result in population level effects.

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