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# EFFECTS OF 7,12-DIMETHYLBENZ[*a*]ANTHRACENE ON IMMUNE FUNCTION AND MIXED-FUNCTION OXYGENASE ACTIVITY IN THE EUROPEAN STARLING

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**Abstract**—Immune function and hepatic MFO activity were examined in adult and nestling starlings administered a synthetic PAH, 7,12-dimethylbenz[*a*]anthracene (DMBA). Methods used to examine the starling immune system included immunopathology, macrophage phagocytosis, lymphocyte blastogenesis to concanavalin A, and hemagglutination of sheep erythrocytes (SRBC). Concomitant investigations of MFO activity were conducted in starlings exposed to DMBA. Ethoxyresorufin *O*-dealkylase (EROD) and pentoxyresorufin *O*-depentylase (PROD) were used as indicators of hepatic MFO activity. Changes in MFO activity were compared to chemically altered immune responses following DMBA exposure. Subcutaneous exposure of adult starlings to 125 mg/kg DMBA resulted in suppression of lymphocyte blastogenesis and antibody production to SRBC. EROD and PROD activity were increased 2.8- and 3.4-fold, respectively. Lymphocyte blastogenesis was impaired in adult starlings orally exposed to 125 mg/kg DMBA. The immune system of nestling starlings exposed orally to 100 mg/kg DMBA was altered, as evidenced by decreased phagocytic ability of macrophages and inhibition of lymphocyte blastogenesis. Oral exposure to DMBA did not induce MFO activity in starlings of either age class. Effects of DMBA on immune function and MFO activity in starlings varied with the age of birds and route and length of chemical exposure.

**Keywords**—European starling 7,12-Dimethylbenz[*a*]anthracene Immunology MFO

## INTRODUCTION

The European starling (*Sturnus vulgaris*) shows promise as an indicator species of chemically induced immune dysfunction in wild birds, due to the relative ease with which studies can be conducted in controlled lab situations as well as under more natural environmental conditions. Although wild birds may be susceptible to immunological injury caused by chemical contaminants released into the environment, immune function measurements of birds in their natural habitat has not been reported. The starling may be a useful model for screening large numbers of phylogenetically related birds in situ. For exploratory research and methods development, starlings can be captured in large, baited funnel traps and kept in captivity. Moreover, research can be conducted on starlings in nesting colonies easily established at localized sites using artificial nest boxes. Birds from these nest-box colonies provide a unique opportunity to study both adult and nestling immune systems. Evidence suggests that young starlings are more vulnerable than adults to many chemicals, including famphur, dicrotophos, and diazinon [1–3]. These studies examined a variety of end points, including mortality and cholinesterase activities, and in all cases young birds were more sensitive to chemical toxicity than older starlings.

Nestlings may be at greater risk of chemical exposure than adults due to their feeding habits. Adult starlings are omnivorous birds, whereas nestling diets consist primarily of soil invertebrates. These invertebrates are in direct contact with any contaminant present in the soil. Starlings forage close to their nests, and ingestion of contaminated invertebrates could result in chronic chemical exposure [3]. Exposure to contaminants via the food chain could cause immune dysfunction in starlings, which could be detected by measuring nestling immune responses. The long-term effects of chemically altered immune responses of nestling starlings is unknown. However, impairment of the immune system of young birds could result in fledglings or adults that lack the vigor of noncompromised animals, leading to increased susceptibility of these birds to pathogens.

In this study, the impacts of an immunotoxic chemical on the immune systems of captive, adult starlings and nestlings from a nest-box colony were explored. Birds were administered a synthetic PAH, 7,12-dimethylbenz[*a*]anthracene (DMBA). This compound was chosen because of its comprehensive immunosuppressive effects in mammals and because it is in the same chemical class as more environmentally pervasive PAHs, such as benzo[*a*]pyrene. Surveying the starling immune system is a novel idea, and supporting literature on the effects of PAHs on the immune system of passerine species is sparse. Therefore, in choosing a chemical that could possibly impair starling immune function, it was prudent to study a compound that deleteriously affects the immune system of other animals. Extensive research with mammalian

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species has provided a comprehensive literature base for DMBA.

In addition to measuring immune responses of starlings exposed to DMBA, the effects of this compound on hepatic MFO activity were investigated. In most mammalian and many avian species, metabolism of xenobiotics, including PAHs, occurs via the cytochrome P450, MFO enzyme system [4,5]. Although induction of these enzymes can be detected with conventional methods, the effects of DMBA on MFO activity in starlings has not been reported. Therefore, concomitant investigations of MFO activity were conducted in starlings exposed to DMBA.

## MATERIALS AND METHODS

### *Animals*

Adult starlings were obtained from the U.S. Department of Agriculture in Washington State. Starlings were housed together in an indoor flight cage (2 × 2 × 3.5 m) at the Wildlife Research Facility, U.S. Environmental Protection Agency (EPA), Environmental Research Laboratory, Corvallis, Oregon. The cage was transected by 2-cm dowel perches, and deionized, reverse-osmosis water was provided from a continuously flowing 5-mm hose into a 5-cm-deep, rectangular, plastic floor pan. Birds were fed ad libitum during their entire captivity from a perch feeder attached to the side of the cage and also from a 10-L ground feeder. The diet consisted of both a game bird maintenance chow (Ralston-Purina, St. Louis, MO) and a protein supplement (Animax®, Ralston-Purina) mixed in a two-to-one ratio. Approximately 1 kg of fresh apples supplemented the diet once a week. Birds resided in the flight cage for at least one month before the experiments.

During the dosing experiments, adult starlings were confined to separate but adjoining individual isolation cages (25 × 25 × 60 cm). Dowel perches and ad libitum food and water were provided throughout each study. Birds were acclimated to these cages for one week before experiments.

### *Sample collection*

Three separate experiments are discussed in this paper. The methods used for each study were mostly identical; therefore, general protocols are outlined before individual experiment discussions. Any anomalous procedures are reported in the context of the specific experiment in which they occurred. If no note is made in the individual study section, it can be assumed that all end points described in this section were measured.

Predose body mass was measured 2 d before each experiment and on each dosing day. Final body mass was recorded on the day of necropsy. Spleens were excised, weighed, and placed into RPMI 1640 tissue culture medium (Gibco BRL, Life Technologies, Inc., Grand Island, NY) with penicillin/streptomycin (100 U/100 mg/ml) (RPMI 1640 + pen/strep) (Gibco), pH 7.2. Spleen mass was reported as ratios of spleen mass to body mass in all experiments. Spleens were kept on ice approximately 2 h until total viable spleen cellularity could be determined. Spleens were processed using the methods of Fairbrother and Fowles [6]. Single-cell suspensions were made by forcing individual spleens through clean nylon mesh with a plastic syringe plunger into RPMI 1640 + pen/strep, pH 7.2. Total viable spleen cell numbers were determined by trypan blue exclusion using a hemocytometer.

In the experiments that included histopathology, liver and spleen slices were removed after the organs were weighed and were placed into phosphate-buffered formalin. Bursas from 15-d-old nestlings were weighed at the time of necropsy and immediately placed in phosphate-buffered formalin. Thymus lobes were not weighed (too inconsistent). Formalin-fixed tissue sections were prepared and mounted onto slides for histopathological examination.

The remaining section of liver was wrapped in aluminum foil and placed into liquid nitrogen for approximately three weeks. Microsomes were then prepared for cytochrome P450 assays.

Blood samples were collected via jugular venipuncture. Up to 2 ml blood was taken from each bird, which was immediately euthanized with carbon dioxide following blood collection. One milliliter of blood was placed into 4.5 ml polypropylene snap-cap tubes (Falcon®, Becton-Dickinson, Lincoln Park, NJ) containing 1 ml RPMI 1640 tissue culture medium + 10% heat inactivated fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT) + pen/strep + 10 mM HEPES (Gibco), pH 7.2. This sample was kept at approximately 25°C for about 1 h until it could be processed for macrophage phagocytosis. The remaining blood (approximately 0.05–1.0 ml) was transferred into 2-ml evacuated glass tubes (Vacutainer®, Becton-Dickinson) containing sodium heparin and kept at approximately 25°C until processing (approximately 1.5 h). From this sample the following end points were measured: packed cell volume, hemoglobin concentration, total peripheral white blood cell (WBC) count, and lymphocyte blastogenesis. Thin film blood smears were made for leukocyte differentiation. After the blood was portioned according to each assay, the remainder was centrifuged for 10 min at 1,500 *g* and plasma was removed. Plasma samples were placed into 1.0-ml serum vials and stored at −20°C for a least one week. From these samples, total plasma protein, plasma alanine aminotransferase (ALT), plasma aspartate aminotransferase (AST), and antibody titers to sheep erythrocytes (SRBC) were measured.

Packed cell volumes were determined using standard methods, as reviewed by Dein [7]. Hemoglobin concentration (grams per deciliter) was determined spectrophotometrically using a commercial hemolysis kit (Cambridge Instruments, Inc., Buffalo, NY) and a hemoglobinometer (Reichert Scientific Instruments, Buffalo, NY) [7].

Total peripheral WBC counts were determined using Natt–Herrick's staining solution [7,8] and a Neubauer hemocytometer. Leukocyte differentiation was performed on blood smears stained with Diff-Quick® (Baxter Healthcare Corp., Miami, FL) and classified according to Dein [7] and Campbell [8]. One hundred cells per slide were counted and the percentage of each cell type recorded.

Total plasma protein, ALT, and AST concentrations were determined with a Gilford (SBA-300) automated spectrophotometer (Ciba-Corning Diagnostics, Oberlin, OH), using procedures and reagents supplied by the manufacturer. Methods

for these assays are described in Fairbrother et al. [9]. Kinetics assays (ALT and AST) were read over a 3-min time interval to verify linearity, and the absorbance was recorded at a time when the change in absorbance was constant. The total protein assay employed the biuret reaction, and absorbance was read at a single time point. Plasma samples were diluted twofold and assayed in duplicate. If the precision between the replicates differed by more than 10%, the sample was reanalyzed. Reference normal and abnormal sera, supplied by Ciba-Corning Diagnostics, were included with each run as a quality control measure for evaluating accuracy.

*Immune function assays*

Leucocytes were separated from whole blood using histopaque-119 (Sigma) in a density gradient centrifugation. Cells were placed into 24-well tissue culture plates (Corning, Inc., Corning, NY) at $4 \times 10^6$ cells per well in a final volume of 1 ml. Plates were incubated at 41°C in 5% $CO_2$ for 48 h, at which time nonadherent cells were removed and phagocytosis testing was performed using modified methods of Ragsdale and Grasso [10]. Yeast cells (*Saccharomyces cerevisiae*) were conjugated with fluorescein isothiocyanate (FITC) and opsonized for 30 min at 41°C with fetal bovine serum, and 50 $\mu$l Hanks balanced salt solution containing $1 \times 10^7$ yeast cells was added to each well. Plates were incubated 15 min; washed twice to remove noningested yeast particles; and examined under an inverted, fluorescent microscope (Nikon Diaphot, Nikon, Inc., Garden City, NJ). Phagocytosis was determined by counting 100 macrophages and recording the number of yeast each macrophage ingested. Results were expressed as percentage of macrophages ingesting 0, 1 to 3, or ≥ 4 yeast [11].

Blastogenesis was performed using a modified whole blood method [12–16] optimized for the starling. Peripheral whole blood was diluted one to 10 with RPMI 1640, and 100-$\mu$l aliquots were dispensed into 96-well, U-bottom microtiter plates (Corning) containing either 0 or 5 $\mu$g per well Concanavalin A (Con A) (Sigma) in 0.1 ml medium. Plates were then incubated for 24 h in a 5% $CO_2$, 41°C humidified atmosphere, followed by a 12-h pulse of 0.05 $\mu$Ci of [$^3$H] thymidine (Amersham, Arlington Heights, IL) in 25 $\mu$l medium. The cells were harvested onto glass-fiber strips for scintillation counting using a 24-well cell harvester (Brandel, Biomedical Research and Development Laboratories, Inc., Gaithersburg, MD). The blastogenic response was expressed as a stimulation index (SI) derived by dividing the mean disintegrations per minute (dpm) for triplicate, mitogen-stimulated wells by the mean dpm for unstimulated wells.

Starlings were inoculated intravenously with $4 \times 10^8$ washed SRBC (Colorado Serum Company, Denver, CO) in 100 $\mu$l sterile 0.85% saline. Because titers to SRBC peak 6 to 7 d after antigen presentation in adult starlings (data not shown), birds in all experiments were challenged with SRBC 6 d before study termination. Hemagglutination (HA) titers to SRBC were measured from heat-inactivated plasma (56°C for 1 h) using adapted microtiter techniques [6,17]. Titers were expressed as the inverse of the highest dilution showing HA.

*Cytochrome P450 assays*

Livers were assayed for total protein content, ethoxyresorufin *O*-dealkylase (EROD), and pentoxyresorufin *O*-depentylase (PROD) activity using the methods of Hofius [18]. EROD analyses were conducted by a modified method of Prough et al. [19], and PROD analyses used the methods of Burke et al. [20]. Both assays were run on a Perkin Elmer LS50 luminescent spectrometer (Perkin Elmer Corp., Norwalk, CT). Protein determinations were made using a bicinchoninic acid assay [21]. Results were expressed as picomoles of substrate hydrolyzed per minute per milligram of protein.

*Preparation and analysis of DMBA dosing solutions*

DMBA (Sigma) dosing solutions were prepared by suspending DMBA powder in warmed corn oil and mixing on a vortex mixer until evenly distributed. The concentrations of the dosing solutions were analyzed by GC (Hewlett Packard 5890 Series II GC with flame ionization detector; Hewlett Packard Co., Palo Alto, CA) in the lab of G. Cobb, The Institute of Wildlife and Environmental Toxicology, Clemson University, Clemson, South Carolina. Reference standards were prepared in the lab from the parent compound (95% purity) and hexane. Three samples from each dosing concentration were run in duplicate. The reported value for each dosing solution was the mean of the six measurements. Dosing solutions were verified to be at least 95% of nominal concentrations, and no DMBA was detected in the control solution.

*Experimental design*

*Experiment 1: Subcutaneous exposure of adult starlings.* Sixteen adult starlings of either sex were randomly assigned to four treatment subgroups ($n = 4$ birds per subgroup). Immediately before dosing, birds were weighed and DMBA solutions were prepared to final concentrations of 0, 1, 10, and 25 mg/ml in corn oil and stored in the dark at 25°C. Birds were injected subcutaneously (sc), using a 27-ga needle and a tuberculin syringe, every other day for 10 d with 0, 1, 10, or 25 mg/kg DMBA (total doses 0, 5, 50, and 125 mg/kg). On day 5, all birds were challenged with $4 \times 10^8$ washed sheep SRBC intravenously (iv), and the study was terminated on day 11, 24 h following the final DMBA injection. Spleens were not prepared for histopathology in this experiment.

*Experiment 2: Oral exposure of adult starlings.* Twenty adult starlings were randomly assigned to four subgroups ($n = 5$ birds per subgroup). Birds were weighed, and DMBA solutions were prepared to final concentrations of 0, 5, 12, and 30 mg/ml. Birds were gavaged with 0, 10, 25, or 60 mg/kg for five consecutive days (total doses 0, 50, 125, 300 mg/kg). On the initial day of dosing, all birds were injected iv with SRBC, and the experiment was terminated on day 6, 24 h following the last gavage. In this experiment, spleens were examined for histopathological changes. After the spleen was weighed and a section put into formalin, the remaining tissue was reweighed and put into RPMI 1640 + pen/strep until viable cellularity could be determined. Insufficient data were acquired from the blastogenesis assay due to technical

problems with the cell harvester. Therefore, to determine the blastogenic response from adult starlings gavaged with DMBA, a second experiment was initiated. Due to a low captive bird population ($n = 8$), the study consisted of only two treatment groups (0 and 125 mg/kg) ($n = 4$ birds per treatment). The dosing regime was identical to the original oral exposure study. Blastogenesis was the only end point measured in this second experiment.

*Experiment 3: Oral exposure of nestling starlings.* Since 1988, 48 starling nest boxes have been erected annually at the Willamette Research Station, U.S. Environmental Protection Agency, Corvallis, Oregon, according to the guidelines of Kendall et al. [22]. For this study, daily monitoring of the boxes started in mid-April 1992, and nest condition, clutch size, and hatch date were recorded. After hatching, nests were monitored every 2 d until experiment initiation. When eggs in 24 nest boxes hatched within a day of each other, one bird from each box was included in the study. Because nine boxes hatched on 1 d (group 1) and 15 boxes hatched on the second day (group 2), a randomized block design was used. Birds were randomly assigned to a treatment of 0, 10, or 100 mg/kg DMBA, such that $n = 3$ birds per treatment from group 1 and $n = 5$ birds per treatment from group 2. When nestlings were 9 d old, the dosing trial began, and the birds were orally gavaged with 0, 2, and 20 mg/kg/d DMBA for five consecutive days (total dose 0, 10, and 100 mg/kg). On the initial dosing day for each group, all birds were challenged iv with SRBC. The study was concluded on day 6, 24 h following the last gavage. Birds were 15 d old at the termination of the study.

### *Statistical analyses*

All statistical calculations were performed using the StatView 512+ statistical program for Apple Macintosh personal computers (BrainPower, Inc., Calabasas, CA). With one exception, DMBA experiments were analyzed by ANOVA. When the overall ANOVA $F$ statistic resulted in $p \leq 0.05$, treatment means were separated and compared to that of the control group by Dunnett's $t$ test. Differences between control and treatment groups were considered significant in all experiments when $p \leq 0.05$. Only two treatment groups were included in the separate experiment conducted to measure the blastogenic response of adult starlings orally exposed to DMBA. Therefore, the results were analyzed using an unpaired Student's $t$ test.

## RESULTS

### *Experiment 1: Subcutaneous exposure of adult starlings*

Subcutaneous exposure of adult starlings to DMBA did not affect body mass at any dose. Liver mass, hemoglobin concentrations, and packed cell volumes also were not affected by DMBA treatment.

DMBA elicited histopathological changes to the liver. Minimal necrosis was evident in the livers from the 125-mg/kg group. Two nodules seen in this group were different from those seen in other groups. One contained macrophage-type cells with occasional cell necrosis and loss of hepatocytes. The second nodule was composed of lymphocytes and plasma cells. Sections from all treatment groups contained lymphoid nodules, and evidence of extramedullary hematopoiesis was prevalent in all tissues.

Although plasma protein concentrations were not significantly different among groups, they increased slightly with increasing DMBA doses. Protein concentrations from birds in the highest treatment group (125 mg/kg) were elevated 16% over control. Increases in plasma ALT and AST activities were dose dependent (Table 1). However, these differences were not statistically significant. The lack of significance may have been due to small sample size ($n = 4$ birds per dose) coupled with increasing variability in the higher dose groups. Log and square root transformations of the data did not make the variability more uniform among treatments.

Administration of 5 mg/kg DMBA did not affect any physiological or immune function parameter measured. At 50 mg/kg, an increase in heterophils ($p \leq 0.05$) caused a significantly higher heterophil/lymphocyte ratio ($p \leq 0.05$) than controls (Table 2). No other changes occurred at this dose.

At 125 mg/kg, DMBA caused a 42% decrease in mean spleen-to-body mass ratios when compared to controls: The mean ratio value from control birds was 1.0 mg/g (±0.1 SE), whereas the value from birds exposed to 125 mg/kg DMBA was 0.6 mg/g (±0.07 SE) ($p \leq 0.05$). Total number of viable spleen cells was 52% lower at this highest dose (Table 2). The overall ANOVA $F$ statistic for this test gave a $p$ value of 0.08, and the Dunnett's $t$ test was not significant at 95%. However, this lack of statistical significance could be attributed to the small sample size.

Peripheral leukocyte numbers were significantly decreased in the 125-mg/kg group ($p \leq 0.05$). Lymphocyte numbers were significantly lower at this dose ($p \leq 0.05$), which caused a subsequent increase in the heterophil/lymphocyte ratio ($p \leq 0.05$) (Table 2).

Macrophage phagocytosis was not significantly affected

Table 1. Blood chemistry (mean ± SE)

| Experiment[a] | Plasma protein (g/dl) | Plasma ALT (U/L; mmol/min/L) | Plasma AST (U/L; mmol/min/L) | Hemoglobin (g/dl) |
|---|---|---|---|---|
| 1. Adult, subcutaneous DMBA | | | | |
| Control | 2.7 (0.4) | 167 (7) | 334 (53) | 15.8 (0.4) |
| 5.0 | 3.0 (0.3) | 174 (14) | 308 (22) | 15.4 (0.2) |
| 50.0 | 3.1 (0.1) | 422 (187) | 1,460 (598) | 16.0 (0.2) |
| 125.0 | 3.1 (0.3) | 685 (340) | 1,004 (487) | 15.8 (0.4) |
| 2. Adult, oral DMBA | | | | |
| Control | 3.3 (0.1) | 151 (20) | 343 (51) | 16.3 (0.5) |
| 50.0 | 3.4 (0.2) | 135 (15) | 276 (18) | 16.8 (0.5) |
| 125.0 | 3.6 (0.3) | 313 (171) | 497 (195) | 16.4 (0.6) |
| 300.0 | 3.7 (0.2) | 328 (103) | 479 (134) | 15.5 (0.5) |
| 3. Nestling, DMBA | | | | |
| Control | 3.6 (0.2) | 363 (72) | NM[b] | 12.3 (0.5) |
| 10.0 | 4.3 (0.3) | 383 (92) | NM | 11.4 (0.4) |
| 100.0 | 3.6 (0.4) | 1,378 (211)[c] | NM | 10.3 (0.3)[c] |

[a]DMBA dosages in milligrams per kilogram.
[b]Not measured.
[c]Significantly different from control ($p \leq 0.05$).

Table 2. Spleen cellularity, total and differential white blood cell (WBC) counts (mean ± SE)

| Experiment[a] | Spleen cellularity[b,c] | Total WBC[c] | Heterophil[c] | Lymphocyte[c] | Monocyte[c] | Eosinophil[c] | H/L ratio[d] |
|---|---|---|---|---|---|---|---|
| 1. Adult, subcutaneous DMBA | | | | | | | |
| Control | 17.3 (1.9) | 5.0 (1.1) | 1.5 (0.3) | 3.0 (0.8) | 0.24 (0.05) | 0.13 (0.07) | 0.51 (0.19) |
| 5.0 | 19.2 (5.6) | 4.4 (0.6) | 1.4 (0.3) | 2.8 (0.5) | 0.20 (0.03) | 0.07 (0.07) | 0.57 (0.15) |
| 50.0 | 20.0 (2.4) | 6.0 (0.8) | 3.4 (0.6)[e] | 2.1 (0.4) | 0.15 (0.03) | 0.32 (0.16) | 1.72 (0.25)[e] |
| 125.0 | 8.2 (1.4)[f] | 2.4 (0.2)[e] | 1.4 (0.2) | 0.8 (0.1)[e] | 0.15 (0.01) | 0.06 (0.02) | 1.90 (0.23)[e] |
| 2. Adult, oral DMBA | | | | | | | |
| Control | 14.1 (4.4) | 4.2 (0.8) | 1.3 (0.3) | 2.6 (0.8) | 0.21 (0.07) | 0.02 (0.02) | 0.83 (0.43) |
| 50.0 | 26.7 (4.2)[e] | 5.9 (1.9) | 1.2 (0.2) | 4.0 (1.6) | 0.30 (0.16) | 0.36 (0.11)[e] | 0.57 (0.29) |
| 125.0 | 14.0 (2.1) | 4.6 (1.3) | 2.1 (1.4) | 2.3 (0.1) | 0.10 (0.22) | 0.09 (0.02) | 0.99 (0.69) |
| 300.0 | 13.5 (5.0) | 5.6 (1.1) | 2.6 (0.3) | 2.6 (0.7) | 0.21 (0.07) | 0.14 (0.06) | 1.50 (0.61) |
| 3. Nestling, DMBA | | | | | | | |
| Control | 48.0 (7.0) | 11.1 (2.3) | 6.2 (1.4) | 3.7 (1.0) | 0.17 (0.03) | 1.14 (0.50) | 2.48 (0.87) |
| 10.0 | 42.0 (8.0) | 9.6 (1.7) | 5.1 (1.4) | 3.7 (0.3) | 0.20 (0.05) | 0.62 (0.18) | 1.40 (0.37) |
| 100.0 | 21.0 (5.0)[e] | 8.1 (1.3) | 4.5 (0.8) | 2.9 (0.8) | 0.14 (0.02) | 0.56 (0.18) | 3.67 (1.54) |

[a]DMBA dosages in milligrams per kilogram.
[b]Number of viable leukocytes per spleen.
[c]Expressed as (cells × $10^{-6}$/ml).
[d]Heterophil/lymphocyte ratios.
[e]Significantly different from control ($p \leq 0.05$).
[f]$p = 0.08$.

in any group; however, Figure 1 displays a salient trend. As DMBA concentrations increased, the percentage of macrophages that ingested <4 yeast increased, with a subsequent decrease in the percentage of macrophages that ingested ≥4 yeast.

Blastogenesis and HA titers were significantly lower in birds dosed with 125 mg/kg DMBA. Mean stimulation indexes were 79% lower from birds at this dose than controls (Fig. 2) ($p \leq 0.05$), and HA titers were decreased 26% in this group. Hemagglutination titers to SRBC were expressed as the $\log_2$ transformation of the data. Therefore, birds receiving no DMBA had HA titers of 10.8 (±0.6 SE), and birds receiving 125 mg/kg DMBA had titers of 8 (±0.7) ($p \leq 0.05$).

A dose-dependent increase was seen in the induction of both EROD and PROD activity in starlings exposed sc to DMBA with statistically significant elevations at the 125-mg/kg dose (Fig. 3). EROD and PROD activity was increased 2.8 and 3.4 times, respectively. Neither enzyme was induced by oral exposure to DMBA in either adult or nestling starlings (Fig. 3).

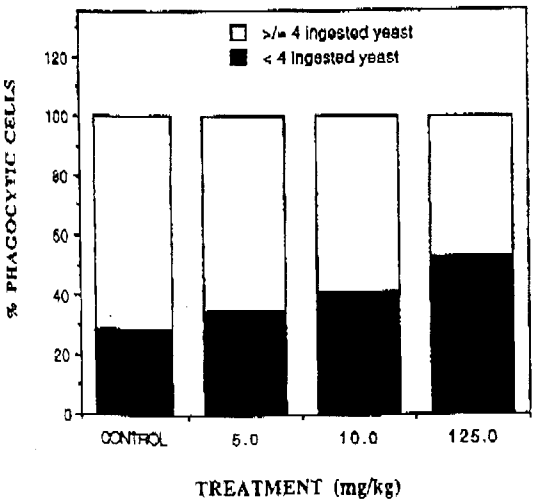


Fig. 1. Macrophage phagocytosis after subcutaneous DMBA exposure of adult starlings. Percentage of macrophages ingesting ≥4 yeast decreases with increasing dosages of DMBA.

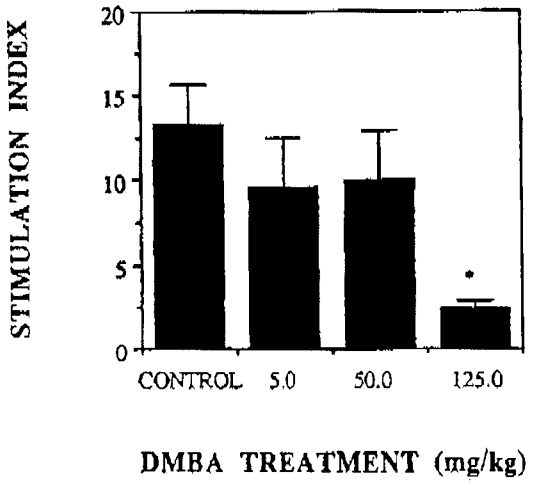


Fig. 2. Blastogenic responses of leukocytes to Con A after subcutaneous exposure of adult starlings to DMBA. Asterisk indicates mean values are significantly different from control ($p \leq 0.05$); bars are 1 SE of mean observations.

*Experiment 2: Oral exposure of adult starlings*

Oral exposure to DMBA did not substantially affect starling physiology or immune function. Histopathological examinations revealed little damage to the livers and no damage to the spleens. Bile duct proliferation was the only lesion associated with increasing dosages of DMBA. No lesions of hepatocytes were seen in any livers.

Although not significantly different from controls, a dose-related increase in the plasma ALT and AST activities was observed as DMBA doses were increased (Table 1). This lack of statistical significance may be due to small sample size and high variability in the data at the 125- and 300-mg/kg doses.

An anomalous finding occurred in the lowest dose (50 mg/kg) group. Spleen-to-body-mass ratios were increased 46% in this group when compared to controls, with a subsequent 47% increase in number of viable spleen cells (Table 2) ($p \leq 0.05$). Eosinophilia was evidenced in this group by a 93% increase in eosinophils over controls (Table 2) ($p \leq 0.05$).

The only other adverse effect noticed in this experiment was a decrease in the blastogenic response at the 125-mg/kg dose of DMBA. The mean stimulation index was 88% lower in the treatment group than in controls (Fig. 4A) ($p \leq 0.05$).

*Experiment 3: Oral exposure of nestling starlings*

At 10 mg/kg DMBA did not exert adverse effects on nestling starlings. However, at 100 mg/kg many physiological and immunological changes occurred in these birds. Body mass and bursa mass were significantly decreased in the 100-mg/kg dose group. Nestlings gavaged only with corn oil had mean body weights of 62 g (±1.8 SE), whereas birds that received 100 mg/kg DMBA had mean weights of 55 g (±2.7 SE) ($p \leq 0.05$). The bursa from control starlings weighed 157.8 mg (±13.5 SE), but the mean weight of bursas from nestlings gavaged with 100 mg/kg was 98.6 (±12.3 SE). Liver weights were not affected in young starlings exposed to DMBA.

Livers, spleens, bursas, and thymuses from 15-d-old starlings were examined histopathologically. DMBA did not cause serious lesions in any examined tissue. Hepatocytes appeared normal in all groups. Bile duct proliferation was

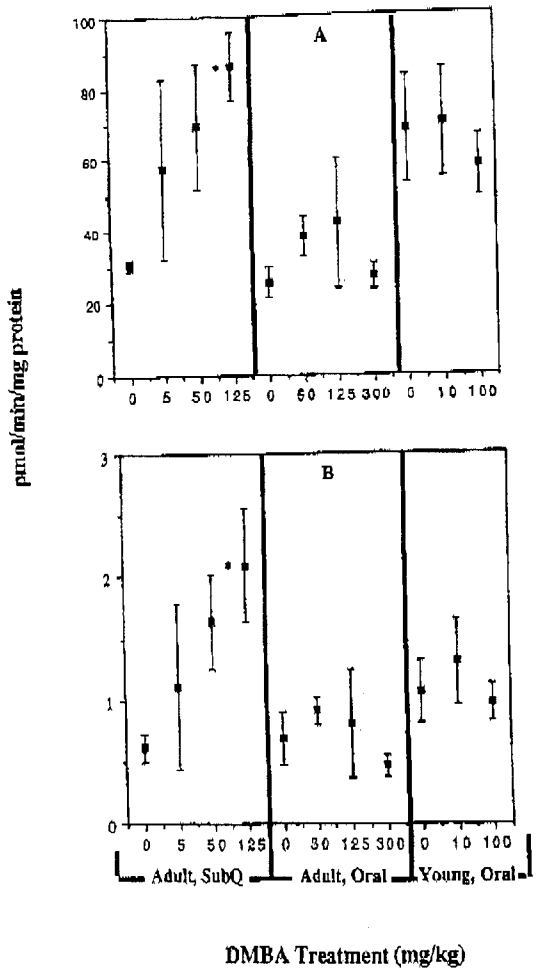


Fig. 3. MFO activities from starlings exposed to DMBA: (A) EROD, (B) PROD. Asterisk indicates significant differences from control values ($p < 0.05$); bars are 1 SE of mean observations.

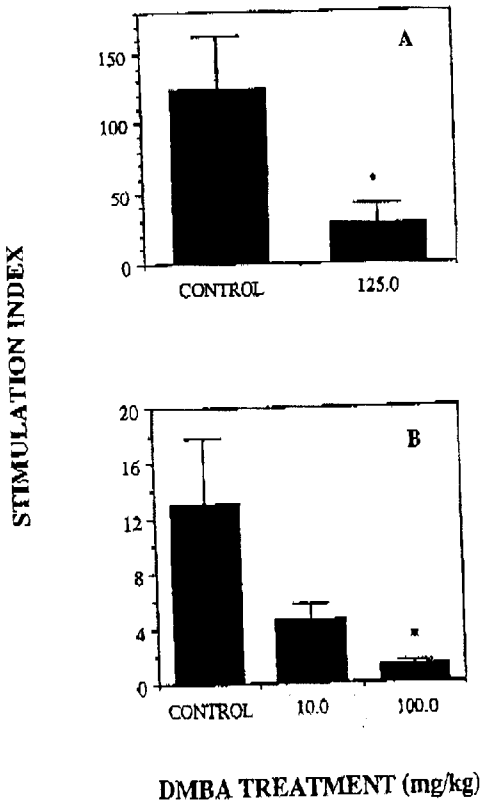


Fig. 4. Blastogenic responses of leukocytes to Con A after oral exposure of starlings to DMBA: (A) adults, (B) nestlings. Asterisk indicates mean values are significantly different from control ($p \leq 0.05$); bars are 1 SE of mean observations.

prominent in five nestlings from the 100-mg/kg group. However, three of the eight birds showed no pronounced proliferation. The thymus, bursa, and spleen appeared normal in all groups. Lymphoid nodules were not prominent in the spleen, suggesting little antigenic stimulation.

Packed cell volumes and plasma protein concentrations were not altered by DMBA exposure. However, mean hemoglobin concentrations were decreased 16% in the 100-mg/kg dose group (Table 1) ($p \leq 0.05$). Mean plasma ALT activity in the 100-mg/kg group was significantly higher than control activity (Table 1) ($p \leq 0.05$).

Although spleen-to-body-mass ratios were not affected by DMBA treatment, mean number of viable spleen cells was significantly lower in the highest DMBA group (Table 2) ($p \leq 0.05$). DMBA did not affect total number of peripheral WBC or alter the distribution of cell type in any group (Table 2).

Lymphocyte proliferation was significantly lower in the 100-mg/kg group (Fig. 4B) ($p \leq 0.05$), with mean stimulation indexes reduced by 90% when compared to controls. Nestling humoral immunity, as measured by HA titers, was not affected by DMBA treatment.

Macrophage phagocytosis was affected by DMBA in a dose-dependent manner (Fig. 5). The percentage of macrophages ingesting ≥4 yeast decreased as the dose of DMBA increased. Therefore, at 100 mg/kg, the phagocytic ability of macrophages was significantly decreased ($p \leq 0.05$).

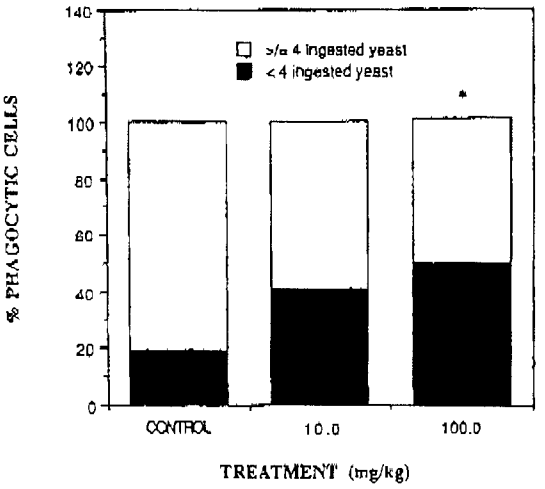


Fig. 5. Macrophage phagocytosis after DMBA exposure of nestling starlings. Asterisk indicates macrophages from control birds ingested significantly more yeast than macrophages from birds administered 100 mg/kg DMBA.

## DISCUSSION

Responses of adult starlings to sc, 10-d exposure of 125 mg/kg DMBA were similar to those reported in mice receiving 100 mg/kg DMBA sc over a two-week period [23]. Lymphocyte proliferation and humoral immune responses were impaired in both mice and starlings. Subcutaneous exposure of 125 mg/kg DMBA may be the threshold dose that results in immunosuppression without causing overt signs of toxicity in starlings. At doses lower than 125 mg/kg (5 and 50 mg/kg), starling immune function was not impaired. In mice, sc administration of 5 mg/kg resulted in suppression of the lymphocyte proliferation response to the T-cell mitogen PHA, but did not affect humoral or nonspecific immune functions [24]. However, in mice exposed to 50 mg/kg DMBA sc over a two-week period, humoral, cell-mediated, and nonspecific immune function end points were inhibited [25–27]. Therefore, starling and mice immune systems respond similarly to sc DMBA treatment, but with different sensitivities.

Macrophage phagocytosis was not significantly affected in starlings exposed sc to DMBA, although an apparent trend in the data indicates a decrease in phagocytosis of macrophages from birds on high doses of DMBA compared to control birds. Yamashita and Hamaoka [28] reported a decrease in antigen-presenting ability by splenic macrophages from mice exposed to a single sc dose of 500 μg DMBA. They speculated that DMBA immunosuppression is due directly to the inhibition of macrophage function. In contrast, Dean et al. [24] suggest that DMBA may directly affect B-cells, because antibody responses to the T-cell-independent, macrophage-independent antigen trinitrophenol-lipopolysaccharide (TNP-LPS) was significantly depressed in mice exposed subcutaneously to 5 to 100 mg/kg DMBA. Further research is needed to determine the mechanism of DMBA immunotoxicity in sc-exposed adult starlings. Antibody responses to T-cell-independent and/or macrophage-independent antigens were not measured, so direct effects of DMBA on starling B-cells could not be determined.

Immunosuppression was minimal in adult starlings gavaged with DMBA, even at doses almost three times greater than concentrations that caused more severe immunosuppression when administered subcutaneously. Proliferation of lymphocytes to Con A was suppressed, but macrophage phagocytosis and the antibody response to SRBC were not altered. Route of exposure appears to have a pronounced influence on DMBA immunotoxicity in starlings. Subcutaneously administered chemicals are usually absorbed at a slower rate than those ingested, as the latter enters the circulatory system more rapidly [29]. DMBA may have been more toxic to sc-exposed birds because of the slower rate of absorption combined with a longer exposure time. Gavaged birds may have been less affected by DMBA because the high metabolic rate of starlings facilitated rapid gastrointestinal motility and excretion of the DMBA-laden corn oil. The dichotomy between the effects of sc and oral routes of exposure also may be due partially to the length of each dosing study. The birds receiving DMBA sc were exposed every other day for 10 d, whereas the orally gavaged birds were dosed for five consecutive days. However, several studies have indicated that length of exposure is of negligible importance when compared to total chemical dose [27,30].

The effects of DMBA administered to young starlings by the oral route were distinctly different from those seen in similarly exposed adults. Overt signs of general toxicity by DMBA were manifested by a decrease in body mass and

blood hemoglobin concentration in young birds exposed to the highest dose (100 mg/kg). Adult starlings exposed to DMBA both subcutaneously and orally did not demonstrate any gross signs of toxicity at similar and greater doses, which suggests that young starlings are more sensitive to DMBA toxicity than adults. Immunosuppression by the carcinogenic PAH benzo[*a*]pyrene is considerably more pronounced in neonatal than in adult mice [31].

The ability of macrophages to ingest fluorescent yeast particles was severely impaired in the nestling high-dose group, as was lymphocyte proliferation. Engulfing and destruction of foreign particles by cells, such as macrophages and granulocytes, is a first line of defense for most animals. The ability of a 15-d-old starling to mount a fully capable immune response is questionable. A lack of secondary follicle development is observed in the spleens of 15-d-old starlings, and the cellular components of the spleen at this age are primarily immature granulocytes and erythrocytes (K.A. Trust, unpublished data). If the starling immune response is not fully developed at 15 d, as indicated by the immaturity of the spleen, the phagocytic and killing ability of nonspecific immune cells may be more important to these birds, and inhibition of this parameter of immunity may have severe consequences.

DMBA caused minimal changes in the livers of starlings, as indicated by histopathological examination. Hepatocellular damage may explain the dose-dependent increases in ALT and AST activity in starlings exposed to DMBA by both routes. In mammals, elevated plasma ALT and AST activity are indicative of soft tissue damage, including the liver. In birds, these enzymes are distributed widely among various tissues, including heart and skeletal muscle, kidneys, and lungs and are therefore not liver specific. However, there have been reports of increased blood ALT values in raptors, chickens, and ducks with hepatic insults, and AST levels are usually greatly increased with liver necrosis, whereas only moderate increases of this enzyme occur with other soft tissue injury [32]. In light of the minimal liver damage caused by DMBA, as revealed by histopathological examination, it is improbable that the increased ALT and AST activities were due solely to hepatic insult and may have been due to damage of other tissues. DMBA did not damage lymphoid organs of adult or nestling starlings.

Induction of both EROD and PROD activities in adult starlings exposed to DMBA sc are similar to findings of Hofius [18], in which oral exposure of nestling starlings to $\beta$-naphthaflavone (BNF) resulted in induction of both enzymes. The lack of induction in nestlings was surprising in light of the fact that Hofius [18] induced P450 activity with similar oral doses of BNF in nestlings. Perhaps the most intriguing aspect of this project, however, was the vastly different results from each experiment regarding the correlation between immunotoxic effects of DMBA and P450 enzyme induction. At the highest dose (125 mg/kg), DMBA caused immunosuppression and induction of hepatic monooxygenase enzymes in sc-exposed adult starlings. At doses almost three times greater (300 mg/kg), DMBA had minimal immunotoxic effects and no increased P450 activity in orally exposed adults. In adults orally exposed to 125 mg/kg DMBA, mitogenically induced T-cell proliferation was decreased. Fifteen-day-old nestlings orally exposed to DMBA (100 mg/kg) were immunosuppressed but had no induction of P450 enzymes. Subcutaneous exposure of DMBA does not induce hepatic monooxygenase activity in either Ah-inducible or noninducible strains of mice, but both types are immunosuppressed [30,33]. As DMBA is a relatively poor inducer of P450 enzymes [34] but a strong immunosuppressant, the relationship between immunosuppression and enzyme induction may be quite different for other compounds or other species. In starlings, induction of hepatic monooxygenase activity by DMBA may be coincidental with immunotoxic effects of the chemical but not necessary for immunosuppression. In vitro, the parent compound appears to be responsible for the immunotoxic effects of DMBA [35].

The parent compound may be immunosuppressive in starlings, but greater hepatic MFO activity in nestlings may increase the immunotoxic effects of DMBA. Control values of EROD activity from the nestling experiment were approximately three times higher than control values from the adult oral experiment. Even though MFO activity was not increased in nestlings exposed to DMBA, constitutively expressed P450 activity is greater in young starlings than in adults. Therefore, nestlings could have metabolized more of the parent compound to toxic products than the orally exposed adults. Alternatively, studies of mice by Twerdok et al. [36] and Ladics et al. [37] have shown that benzo[*a*]pyrene-diol is converted to the reactive intermediate, 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene intracellularly by neutrophils and macrophages, respectively. If this is true, then the observed differences in toxic responses between orally dosed juveniles and sc-dosed adults would be related to differing doses at the cellular level rather than enzyme activity differences in the liver. However, the enzymatic content and cytochemical reactions of avian heterophils differ from the mammalian neutrophil analog [38]. The mammalian neutrophil relies on myeloperoxidase–halide interactions to facilitate actual killing, whereas the activity of heterophils is primarily associated with cationic proteins and lysozymes within the granules. The ability of avian heterophils to activate compounds such as DMBA is unknown. Therefore, it currently is not possible to determine if the greater sensitivity of nestling starlings to DMBA was due to the higher activity of nestling liver cytochromes or to differences in heterophil/macrophage activation as a result of the different exposure routes.

The main objective of this project was to explore the starling immune system as a potential indicator of avian exposure to environmental contaminants. Starling immune function, both adult and nestling, can be measured, and chemical insults to the system can be detected. Flightless nestlings make ideal test animals because they are easily handled and unable to leave the nest box. Although further research is necessary to develop a more rigorous assay for humoral responses to quantify actual effects of chemicals on starling B-cells, the immunological battery of tests used in this project can be incorporated into research examining the effects of chemicals on other biochemical and physiological responses of star-

lings, such as MFO activity. This holistic approach could provide more comprehensive information about the effects of toxicants on avian systems.

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